(54) Title: HLA BINDING PEPTIDES AND THEIR USES

(57) Abstract

The present invention provides peptide compositions capable of specifically binding selected MHC alleles and inducing T cell activation in T cells restricted by the MHC allele. The peptides are useful to elicit an immune response against a desired antigen.

<u>HLA BINDING PEPTIDES AND THEIR USES</u>

The present application is a continuation in part of USSN 08/027,746 which is a continuation in part of USSN 07/926,666, which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as viral diseases and cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and inducing an immune response.

MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed. Class I MHC molecules are expressed on almost all nucleated cells and are recognized by cytotoxic T lymphocytes (CTLs), which then destroy the antigen-bearing cells. CTLs are particularly important in tumor rejection and in fighting viral infections. The CTL recognizes the antigen in the form of a peptide fragment bound to the MHC class I molecules rather than the intact foreign antigen itself. The antigen must normally be endogenously synthesized by the cell, and a portion of the protein antigen is degraded into small peptide fragments in the cytoplasm. Some of these small peptides translocate into a pre-Golgi compartment and interact with class I heavy chains to facilitate proper folding and association with the subunit ß2 micr gl bulin. The peptide-MHC class I c mplex is then r uted t the cell surface for expressi n and potential recogniti n by specific CTLs.

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Investigations of the crystal structure of the human MHC class I molecule, HLA-A2.1, indicate that a peptide binding groove is created by the folding of the α1 and α2 domains of the class I heavy chain (Bjorkman et al., _Nature_ 329:506 (1987). In these investigations, however, the identity of peptides bound to the groove was not determined.

Buus et al., _Science_ 242:1065 (1988) first described a method for acid elution of bound peptides from MHC. Subsequently, Rammensee and his coworkers (Falk et al., _Nature_ 351:290 (1991) have developed an approach to characterize naturally processed peptides bound to class I molecules. Other investigators have successfully achieved direct amino acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from class I molecules of the B type (Jardetzky, et al., _Nature_ 353:326 (1991) and of the A2.1 type by mass spectrometry (Hunt, et al., _Science_ 225:1261 (1992). A review of the characterization of naturally processed peptides in MHC Class I has been presented by Rötzschke and Falk (Rötzschke and Falk, _Immunol. Today_ 12:447 (1991).

Sette et al., _Proc. Natl. Acad. Sci. USA_ 86:3296 (1989) showed that MHC allele specific motifs could be used to predict MHC binding capacity. Schaeffer et al., _Proc. Natl. Acad. Sci. USA_ 86:4649 (1989) showed that MHC binding was related to immunogenicity. Several authors (De Bruijn et al., _Eur. J. Immunol._, 21:2963-2970 (1991); Pamer et al., 991 _Nature_ 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Class I motifs specific for a number of human alleles of a given class I isotype have yet to be described. It is desirable that the combined frequencies of these different alleles should be high enough to cover a large fraction or perhaps the majority of the human outbred population.

Despite the developments in the art, the prior art has y t t pr vide a useful human peptide-based vaccin   r therapeutic agent based on this work. The present invention provides these and other advantages.

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SUMMARY OF THE INVENTION

The present inventi n provides compositions comprising immunogenic peptides having binding motifs for MHC Class I molecules. The immunogenic peptides are typically between about 8 and about 11 residues and comprise conserved residues involved in binding proteins encoded by the appropriate MHC allele. A number of allele specific motifs have been identified.

For instance, the motif for HLA-A3.2 comprises from the N-terminus to C-terminus a first conserved residue of L, M, I, V, S, A, T and F at position 2 and a second conserved residue of K, R or Y at the C-terminal end. Other first conserved residues are C, G or D and alternatively E. Other second conserved residues are H or F. The first and second conserved residues are preferably separated by 6 to 7 residues.

The motif for HLA-A1 comprises from the N-terminus to the C-terminus a first conserved residue of T, S or M, a second conserved residue of D or E, and a third conserved residue of Y. Other second conserved residues are A, S or T. The first and second conserved residues are adjacent and are preferably separated from the third conserved residue by 6 to 7 residues. A second motif consists of a first conserved residue of E or D and a second conserved residue of Y where the first and second conserved residues are separated by 5 to 6 residues.

The motif for HLA-A11 comprises from the N-terminus to the C-terminus a first conserved residue of T or V at position 2 and a C-terminal conserved residue of K. The first and second conserved residues are preferably separated by 6 or 7 residues.

The motif for HLA-A24.1 comprises from the N-terminus to the C-terminus a first conserved residue of Y, F or W at position 2 and a C terminal conserved residue of F, I, W, M or L. The first and second conserved residues are preferably separated by 6 to 7 residues.

Epitop s n a number of potential target pr teins can be identified in this manner. Examples of suitable

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antigens include prostate specific antigen (PSA), hepatitis B
core and surfac antigens (HBVc, HBVs) hepatitis C antigens,
malignant melanoma antigen (MAGE-1) Epstein-Barr virus
antigens, human immunodeficiency type-1 virus (HIV1) and
5   papilloma virus antigens. The peptides are thus useful in
pharmaceutical compositions for both _in vivo_ and _ex vivo_
therapeutic and diagnostic applications.

Definitions
10   The term "peptide" is used interchangeably with
"oligopeptide" in the present specification to designate a
series of residues, typically L-amino acids, connected one to
the other typically by peptide bonds between the alpha-amino
and carbonyl groups of adjacent amino acids. The
15   oligopeptides of the invention are less than about 15 residues
in length and usually consist of between about 8 and about 11
residues, preferably 9 or 10 residues.
         An "immunogenic peptide" is a peptide which
comprises an allele-specific motif such that the peptide will
20   bind the MHC allele and be capable of inducing a CTL response.
Thus, immunogenic peptides are capable of binding to an
appropriate class I MHC molecule and inducing a cytotoxic T
cell response against the antigen from which the immunogenic
peptide is derived.
25   A "conserved residue" is an amino acid which occurs
in a significantly higher frequency than would be expected by
random distribution at a particular position in a peptide
motif. Typically a conserved residue is one at which the
immunogenic peptide may provide a contact point with the MHC
30   molecule. One to three, preferably two, conserved residues
within a peptide of defined length defines a motif for an
immunogenic peptide. These residues are typically in close
contact with the peptide binding groove, with their side
chains buried in specific pockets of the groove itself.
35   Typically, an immunogenic peptide will comprise up t three
c nserved residues, m re usually two c nserv d residues.
         As used herein, "negative binding residues" are
amino acids which if present at certain positions will result

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in a peptide b ing a nonbinder r po r binder and in turn fail
to induce a CTL resp ns despite th presence of the
appropriate conserved residues within the peptide.

     The term "motif" refers to the pattern of residues
5   in a peptide of defined length, usually about 8 to about 11
amino acids, which is recognized by a particular MHC allele.
The peptide motifs are typically different for each human MHC
allele and differ in the pattern of the highly conserved
residues.

10   The binding motif for an allele can be defined with
increasing degrees of precision. In one case, all of the
conserved residues are present in the correct positions in a
peptide and there are no negative binding residues present.

     The phrases "isolated" or "biologically pure" refer
15   to material which is substantially or essentially free from
components which normally accompany it as found in its native
state. Thus, the peptides of this invention do not contain
materials normally associated with their _in situ_ environment,
e.g., MHC I molecules on antigen presenting cells. Even where
20   a protein has been isolated to a homogenous or dominant band,
there are trace contaminants in the range of 5-10% of native
protein which co-purify with the desired protein. Isolated
peptides of this invention do not contain such endogenous co-
purified protein.

25   The term "residue" refers to an amino acid or amino
acid mimetic incorporated in an oligopeptide by an amide bond
or amide bond mimetic.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification
scheme.

5   Fig. 2 is an SDS-PAGE analysis of affinity purified.
HLA-A3.2 from the cell line EHM using an affinity column
prepared with the mAb GAP A3 coupled to protein A-Sepharose.

Lane 1 - Molecular weight standards.

Lane 2 - A3.2 acid eluate

10   Lane 3 - A3.2 a second acid eluate

Lane 4 - Base elution #1

Lane 5 - Base elution #2

Lane 6 - Concentrated base elution 1

Lane 7 - Concentrated base elution 2

15   Lane 8 - BSA - 10 μg

Lane 9 - BSA - 3 μg

Lane 10 - BSA - 1 μg

Fig. 3 shows reverse phase high performance liquid
chromatography (RP-HPLC) separation of HLA-A3 acid eluted

20 peptides.

Fig. 4 shows binding of a radioactively labeled
peptide of the invention to MHC molecules as measured by the %
bound radioactivity.

Fig. 5 shows inhibition of binding of a peptide of

25 the invention to MHC molecules in the presence of three
peptides [HBc 18-27 (924.07), a Prostate Specific Antigen
peptide (939.01), and HIV nef 73-82 (940.03)].

Fig. 6 shows the dependency of the binding on MHC
concentration in the presence or absence of β_2 microglobulin.

30   Fig. 7 shows dose dependent inhibition of binding
with the addition of unlabeled peptide.

Fig. 8 Scatchard Analysis of binding to MHC A11
confirming an apparent K_D of 6nM.

Fig. 9 shows the binding of a radioactively labeled

35 peptide of the invention to MHC A1 as measured by % bound
r activity.

Fig. 10 shows dose dependent inhibition of binding
with the addition of unlabeled peptide.

Fig. 11 Scatchard Analysis of binding to MHC A1
c nfirming an appar nt K_D f 21nM.

Fig. 12 sh ws the binding of two peptides f this
invention as a function of MHC A24 concentration as measured
by % bound reactivity.

Fig. 13 shows the dose dependent inhibition of
binding to MHC A24 with the addition of unlabeled peptides.

Figs. 14(a) and 14(b) show the Scatchard Analysis of
binding to MHC A24 of the two peptides confirming a K_D of 30
and 60nM, respectively.

Fig. 15 shows the effect on MHC class 1 molecules of
$\beta_2$microglobulin and a peptide of choice on acid-stripped PHA
blasts.

Fig. 16 shows CTL induction using GC43 A2.1
responders and autologous acid-stripped PBMCs or PHA blasts
loaded with the 777.03-924.07-927.32 peptide pool.

Fig. 17 shows CTL induction using X351 or X355 A2.1
responders and autologous acid stripped PBMCs or PHA blasts as
stimulators after loading with the 1044.04-1044.05-1044.06
peptide pool.

Fig. 18 shows CTL induction using GC49 A2.1
responders and Autologous Acid stripped PHA blasts as
stimulators after loading with 939.03 peptide.

Fig. 19 shows CTL induction using GC66 A1 responders
and autologous acid stripped PBMCs as stimulators after
loading of peptide 938.01.

Fig. 20 illustrates the lysis of peptide sensitized
targets and endogenous targets following stimulation with SAC-
I activated PBMCs loaded with a MAGE 3 peptide.

Fig. 21 shows a comparison of the acid strip loading
with the cold temperature incubation.

Fig. 22 shows a CTL response to an immunogenic
peptide for MAGE/A11.

Fig. 23 shows a CTL response to an immunogenic
peptide for HIV/A3.

Fig. 24 sh ws a CTL respons to an immun genic
peptide for HCV/A3.

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Fig. 25 shows a CTL response to an immunogenic peptide for HBV/A1.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

5   The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral
10   diseases or cancers, for which the amino acid sequence of the potential antigen targets is known.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B
15   core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

Peptides comprising these epitopes are synthesized
20   and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I
25   assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary _in vitro_ or _in_
30   _vivo_ CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell
35   surface at approximately equal densities, whereas the expressi n of HLA-C is significantly l wer (perhaps as much as 10-f ld lower). Each of these loci have a number of alleles.

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The peptide binding motifs f the inventi n are relatively
specific f r each allelic subtype.

For peptide-based vaccines, the peptides of the
present invention preferably comprise a motif recognized by an
5   MHC I molecule having a wide distribution in the human
population. Since the MHC alleles occur at different
frequencies within different ethnic groups and races, the
choice of target MHC allele may depend upon the target
population. Table 1 shows the frequency of various alleles at
10   the HLA-A locus products among different races. For instance,
the majority of the Caucasoid population can be covered by
peptides which bind to four HLA-A allele subtypes,
specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the
majority of the Asian population is encompassed with the
15   addition of peptides binding to a fifth allele HLA-A11.2.

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TABLE 1

A Allele/Subtype	N(69)*	A(54)	C(502)
A1	10.1(7)	1.8(1)	27.4(138)
A2.1	11.5(8)	37.0(20)	39.8(199)
A2.2	10.1(7)	0	3.3(17)
A2.3	1.4(1)	5.5(3)	0.8(4)
A2.4	–	–	–
A2.5	–	–	–
A3.1	1.4(1)	0	0.2(0)
A3.2	5.7(4)	5.5(3)	21.5(108)
A11.1	0	5.5(3)	0
A11.2	5.7(4)	31.4(17)	8.7(44)
A11.3	0	3.7(2)	0
A23	4.3(3)	–	3.9(20)
A24	2.9(2)	27.7(15)	15.3(77)
A24.2	–	–	–
A24.3	–	–	–
A25	1.4(1)	–	6.9(35)
A26.1	4.3(3)	9.2(5)	5.9(30)
A26.2	7.2(5)	–	1.0(5)
A26V	–	3.7(2)	–
A28.1	10.1(7)	–	1.6(8)
A28.2	1.4(1)	–	7.5(38)
A29.1	1.4(1)	–	1.4(7)
A29.2	10.1(7)	1.8(1)	5.3(27)
A30.1	8.6(6)	–	4.9(25)
A30.2	1.4(1)	–	0.2(1)
A30.3	7.2(5)	–	3.9(20)
A31	4.3(3)	7.4(4)	6.9(35)
A32	2.8(2)	–	7.1(36)
Aw33.1	8.6(6)	–	2.5(13)
Aw33.2	2.8(2)	16.6(9)	1.2(6)
Aw34.1	1.4(1)	–	–
Aw34.2	14.5(10)	–	0.8(4)
Aw36	5.9(4)	–	–

Table compiled from B. DuPont, _Immunobiology of HLA_, Vol. I, Histocompatibility Testing 1987, Springer-Verlag, New York 1989.

* N – negroid; A = Asian; C = caucasoid. Numbers in parenthesis represent the number of individuals included in the analysis.

The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would

assum at physiologic pH values, unless otherwis specified.
In the amin acid structure formulae, each residue is
generally represented by standard three letter or single
letter designations. The L-form of an amino acid residue is
represented by a capital single letter or a capital first
letter of a three-letter symbol, and the D-form for those
amino acids is represented by a lower case single letter or a
lower case three letter symbol. Glycine has no asymmetric
carbon atom and is simply referred to as "Gly" or G.

        The procedures used to identify peptides of the
present invention generally follow the methods disclosed in
Falk et al., *Nature* 351:290 (1991), which is incorporated
herein by reference. Briefly, the methods involve large-scale
isolation of MHC class I molecules, typically by
immunoprecipitation or affinity chromatography, from the
appropriate cell or cell line. Examples of other methods for
isolation of the desired MHC molecule equally well known to
the artisan include ion exchange chromatography, lectin
chromatography, size exclusion, high performance ligand
chromatography, and a combination of all of the above
techniques.

        A large number of cells with defined MHC molecules,
particularly MHC Class I molecules, are known and readily
available. For example, human EBV-transformed B cell lines
have been shown to be excellent sources for the preparative
isolation of class I and class II MHC molecules.
Well-characterized cell lines are available from private and
commercial sources, such as American Type Culture Collection
("Catalogue of Cell Lines and Hybridomas," 6th edition (1988)
Rockville, Maryland, U.S.A.); National Institute of General
Medical Sciences 1990/1991 Catalog of Cell Lines (NIGMS) Human
Genetic Mutant Cell Repository, Camden, NJ; and ASHI
Repository, Bingham and Women's Hospital, 75 Francis Street,
Boston, MA 02115. Table 2 lists some B cell lines suitable
for use as s urces for HLA-A all les. All f these cell lines
can be grown in large batches and are theref re useful f r
large scale producti n of MHC molecul s. One f skill will
recognize that these ar merely exemplary cell lines and that

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many other cell sources can be employed. Similar EBV B cell
lines hom zyg us for HLA-B and HLA-C could serve as sourc s
for HLA-B and HLA-C alleles, respectively.

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TABLE 2

HUMAN CELL LINES (HLA-A SOURCES)

HLA-A allele	B cell line
A1	MAT COX (9022) STEINLIN (9087)
A2.1	JY
A3.2	EHM (9080) HO301 (9055)GM3107
A24.1	KT3(9107),TISI (9042)
A11	BVR (GM6828A) WT100 (GM8602),WT52 (GM8603)

In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used. For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B, and HLA-C molecules. Several mAb reagents for the isolation of HLA-A molecules are available (Table 3). Thus, for each of the targeted HLA-A alleles, reagents are available that may be used for the direct isolation of the HLA-A molecules. Affinity columns prepared with these mAbs using standard t chniques are successfully used to purify the respective HLA-A allele products.

In addition to allel -specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and

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one anti-HLA-B, C mAb, Bl.23.2, could be us d in alternative
affinity purification pr tocols as described in the example
secti n below.

5   TABLE 3

                          ANTIBODY REAGENTS

10

anti-HLA	Name
HLA-A1	12/18
HLA-A3	GAPA3 (ATCC, HB122)
HLA-11,24.1	A11.1M (ATCC, HB164)
HLA-A,B,C	W6/32 (ATCC, HB95)
monomorphic	B9.12.1 (INSERM-CNRS)
HLA-B,C	B.1.23.2 (INSERM-CNRS)
monomorphic	

35   The peptides bound to the peptide binding groove of
the isolated MHC molecules are eluted typically using acid
treatment. Peptides can also be dissociated from class I
molecules by a variety of standard denaturing means, such as
heat, pH, detergents, salts, chaotropic agents, or a
40 combination thereof.
            Peptide fractions are further separated from the MHC
molecules by reversed-phase high performance liquid
chromatography (HPLC) and sequenced. Peptides can be
separated by a variety of other standard means well known to
45 the artisan, including filtration, ultrafiltration,
  lectr phor sis, size chr matography, precipitati n with
sp cific antibodies, ion exchange chromatography,
isoelectrofocusing, and the like.

Sequ ncing f the isolated peptides can be perf rmed
according t standard t chniques such as Edman degradation
(Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]).
Other methods suitable for sequencing include mass
spectrometry sequencing of individual peptides as previously
described (Hunt, et al., Science 225:1261 (1992), which is
incorporated herein by reference). Amino acid sequencing of
bulk heterogenous peptides (e.g., pooled HPLC fractions) from
different class I molecules typically reveals a characteristic
sequence motif for each class I allele.

Definition of motifs specific for different class I
alleles allows the identification of potential peptide
epitopes from an antigenic protein whose amino acid sequence
is known. Typically, identification of potential peptide
epitopes is initially carried out using a computer to scan the
amino acid sequence of a desired antigen for the presence of
motifs. The epitopic sequences are then synthesized. The
capacity to bind MHC Class molecules is measured in a variety
of different ways. One means is a Class I molecular binding
assay as described in Example 10, below. Other alternatives
described in the literature include inhibition of antigen
presentation (Sette, et al., J. Immunol. 141:3893 (1991), in
vitro assembly assays (Townsend, et al., Cell 62:285 (1990),
and FACS based assays using mutated ells, such as RMA.S
(Melief, et al., Eur. J. Immunol. 21:2963 [1991]).

Next, peptides that test positive in the MHC class I
binding assay are assayed for the ability of the peptides to
induce specific CTL responses in vitro. For instance,
antigen-presenting cells that have been incubated with a
peptide can be assayed for the ability to induce CTL responses
in responder cell populations. Antigen-presenting cells can
be normal cells such as peripheral blood mononuclear cells or
dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987);
Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are
deficient in their ability to load class I m lecules with
internally pr cessed peptides, such as the mouse cell lines
RMA-S (Kärre, t al.. Nature, 319:675 (1986); Ljunggren, et

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al., <u>Eur. J. Immunol</u>. 21:2963-2970 (1991)), and the human
somatic T cell hybridoma, T-2 (Cerundolo, et al., <u>Nature</u>
345:449-452 (1990)) and which hav been transfected with the
appropriate human class I genes are conveniently used, when
5   peptide is added to them, to test for the capacity of the
peptide to induce <u>in vitro</u> primary CTL responses. Other
eukaryotic cell lines which could be used include various
insect cell lines such as mosquito larvae (ATCC cell lines CCL
125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851),
10   armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila
cell lines such as a Schneider cell line (see Schneider <u>J.</u>
<u>Embryol. Exp. Morphol.</u> 27:353-365 [1927]). That have been
transfected with the appropriate human class I MHC allele
encoding genes and the human B_2 microglobulin genes.
15   Peripheral blood lymphocytes are conveniently
isolated following simple venipuncture or leukapheresis of
normal donors or patients and used as the responder cell
sources of CTL precursors. In one embodiment, the appropriate
antigen-presenting cells are incubated with 10-100 μM of
20   peptide in serum-free media for 4 hours under appropriate
culture conditions. The peptide-loaded antigen-presenting
cells are then incubated with the responder cell populations
<u>in vitro</u> for 7 to 10 days under optimized culture conditions.
Positive CTL activation can be determined by assaying the
25   cultures for the presence of CTLs that kill radiolabeled
target cells, both specific peptide-pulsed targets as well as
target cells expressing endogenously processed form of the
relevant virus or tumor antigen from which the peptide
sequence was derived.
30   Specificity and MHC restriction of the CTL is
determined by testing against different peptide target cells
expressing appropriate or inappropriate human MHC class I.
The peptides that test positive in the MHC binding assays and
give rise to specific CTL responses are referred to herein as
35   immunogenic peptides.
                The immun genic peptides can b prepared
synth tically, or by recombinant DNA technol gy or isolated
from natural sources such as whole viruses or tum rs.

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Although the peptide will preferably be substantially free f
 ther naturally occurring h st cell proteins and fragments
 thereof, in some embodiments the peptides can be synthetically
conjugated to native fragments or particles. The polypeptides
5   or peptides can be a variety of lengths, either in their
neutral (uncharged) forms or in forms which are salts, and
either free of modifications such as glycosylation, side chain
oxidation, or phosphorylation or containing these
modifications, subject to the condition that the modification
10 not destroy the biological activity of the polypeptides as
herein described.

Desirably, the peptide will be as small as possible
while still maintaining substantially all of the biological
activity of the large peptide. When possible, it may be
15 desirable to optimize peptides of the invention to a length of
9 or 10 amino acid residues, commensurate in size with
endogenously processed viral peptides or tumor cell peptides
that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified
20 as necessary to provide certain desired attributes, e.g.,
improved pharmacological characteristics, while increasing or
at least retaining substantially all of the biological
activity of the unmodified peptide to bind the desired MHC
molecule and activate the appropriate T cell. For instance,
25 the peptides may be subject to various changes, such as
substitutions, either conservative or non-conservative, where
such changes might provide for certain advantages in their
use, such as improved MHC binding. By conservative
substitutions is meant replacing an amino acid residue with
30 another which is biologically and/or chemically similar, e.g.,
one hydrophobic residue for another, or one polar residue for
another. The substitutions include combinations such as Gly,
Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys,
Arg; and Phe, Tyr. The effect of single amino acid
35 substituti ns may also be pr bed using D-amino acids. Such
m dificati ns may be made using well known peptid synthesis
pr cedures, as described in e.g., Merrifield, _Science_ 232:341-
347 (1986), Barany and Merrifield, _The Peptides_, Gr ss and

Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, <u>Solid Phase peptide Synthesis</u>, (R ckf rd, Ill., Pierc), 2d Ed. (1984), incorporated by reference herein.

5   The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated

10   that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins,

15   such as L-α-amino acids, or their D-isomers, but may include non-natural amino acids as well, such as β-γ-δ-amino acids, as well as many derivatives of L-α-amino acids.

          Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of

20   electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T

25   cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing

30   necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide.

35   In any event, such substitutions should employ amino acid residues r ther molecular fragments chosen to av id, f r exampl , steric and charge interference which might disrupt binding.

19

Amin acid substitutions are typically f single
residues. Substitutions, deleti ns, inserti ns or any
c mbinati n there f may be combined to arrive at a final
peptide. Substitutional variants are those in which at least
5   one residue of a peptide has been removed and a different
residue inserted in its place. Such substitutions generally
are made in accordance with the following Table 4 when it is
desired to finely modulate the characteristics of the peptide.

20

TABLE 4

Original Residue	Exemplary Substitution
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

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Substantial changes in functi n (e.g., affinity f r
MHC m lecules r T cell recept rs) are made by selecting
substitutions that are less conservative than those in Table
4, i.e., selecting residues that differ more significantly in
their effect on maintaining (a) the structure of the peptide
backbone in the area of the substitution, for example as a
sheet or helical conformation, (b) the charge or
hydrophobicity of the molecule at the target site or (c) the
bulk of the side chain. The substitutions which in general
are expected to produce the greatest changes in peptide
properties will be those in which (a) hydrophilic residue,
e.g. seryl, is substituted for (or by) a hydrophobic residue,
e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a
residue having an electropositive side chain, e.g., lysyl,
arginyl, or histidyl, is substituted for (or by) an
electronegative residue, e.g. glutamyl or aspartyl; or (c) a
residue having a bulky side chain, e.g. phenylalanine, is
substituted for (or by) one not having a side chain, e.g.,
glycine.

        The peptides may also comprise isosteres of two or
more residues in the immunogenic peptide. An isostere as
defined here is a sequence of two or more residues that can be
substituted for a second sequence because the steric
conformation of the first sequence fits a binding site
specific for the second sequence. The term specifically
includes peptide backbone modifications well known to those
skilled in the art. Such modifications include modifications
of the amide nitrogen, the α-carbon, amide carbonyl, complete
replacement of the amide bond, extensions, deletions or
backbone crosslinks. See, generally, Spatola, Chemistry and
Biochemistry of Amino Acids, peptides and Proteins, Vol. VII
(Weinstein ed., 1983).

        Modifications of peptides with various aminc acid
mimetics or unnatural amino acids are particularly useful in
increasing th stability f the peptide in vivo. Stability
can be assayed in a number f ways. For instance, peptidases
and vari us bi l gical media, such as human plasma and serum,
have been us d to test stability. See, e.g., Verhoef t al.,

Eur. J. Drug Metab Pharmacokin. 11:291-302 (1986). Half life
of the peptides of the present invention is conveniently
determined using a 25% human serum (v/v) assay. The protocol
is generally as follows. Pooled human serum (Type AB,

5   non-heat inactivated) is delipidated by centrifugation before
use. The serum is then diluted to 25% with RPMI tissue
culture media and used to test peptide stability. At
predetermined time intervals a small amount of reaction
solution is removed and added to either 6% aqueous

10   trichloracetic acid or ethanol. The cloudy reaction sample is
cooled (4°C) for 15 minutes and then spun to pellet the
precipitated serum proteins. The presence of the peptides is
then determined by reversed-phase HPLC using
stability-specific chromatography conditions.

15   The peptides of the present invention or analogs
thereof which have CTL stimulating activity may be modified to
provide desired attributes other than improved serum half
life. For instance, the ability of the peptides to induce CTL
activity can be enhanced by linkage to a sequence which

20   contains at least one epitope that is capable of inducing a T
helper cell response. Particularly preferred immunogenic
peptides/T helper conjugates are linked by a spacer molecule.
The spacer is typically comprised of relatively small, neutral
molecules, such as amino acids or amino acid mimetics, which

25   are substantially uncharged under physiological conditions.
The spacers are typically selected from, e.g., Ala, Gly, or
other neutral spacers of nonpolar amino acids or neutral polar
amino acids. It will be understood that the optionally
present spacer need not be comprised of the same residues and

30   thus may be a hetero- or homo-oligomer. When present, the
spacer will usually be at least one or two residues, more
usually three to six residues. Alternatively, the CTL peptide
may be linked to the T helper peptide without a spacer.
        The immunogenic peptide may be linked to the T helper

35   peptide either directly or via a spacer either at the amino r
carboxy terminus f the CTL peptide. The amino terminus of
either the immunogenic peptide or the T helper peptide may be
acylated.

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In s me embodiments it may be desirable to include in
the pharmaceutical compositions f the inventi n at least on
component which assists in priming CTL. Lipids have been
identified as agents capable of assisting the priming CTL *in*
5   *vivo* against viral antigens. For example, palmitic acid
residues can be attached to the alpha and epsilon amino groups
of a Lys residue and then linked, e.g., via one or more
linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the
like, to an immunogenic peptide. The lipidated peptide can
10   then be injected directly in a micellar form, incorporated
into a liposome or emulsified in an adjuvant, e.g., incomplete
Freund's adjuvant. In a preferred embodiment a particularly
effective immunogen comprises palmitic acid attached to alpha
and epsilon amino groups of Lys, which is attached via
15   linkage, e.g., Ser-Ser, to the amino terminus of the
immunogenic peptide.

     As another example of lipid priming of CTL responses,
E. coli lipoproteins, such as
tripalmitoyl-S-glycerylcysteinlyseryl-serine (P$_3$CSS) can be
20   used to prime virus specific CTL when covalently attached to
an appropriate peptide. See, Deres et al., *Nature* 342:561-564
(1989), incorporated herein by reference. Peptides of the
invention can be coupled to P$_3$CSS, for example, and the
lipopeptide administered to an individual to specifically
25   prime a CTL response to the target antigen. Further, as the
induction of neutralizing antibodies can also be primed with
P$_3$CSS conjugated to a peptide which displays an appropriate
epitope, the two compositions can be combined to more
effectively elicit both humoral and cell-mediated responses to
30   infection.

     In addition, additional amino acids can be added to
the termini of a peptide to provide for ease of linking
peptides one to another, for coupling to a carrier support, or
larger peptide, for modifying the physical or chemical
35   properties f the peptid r oligopeptide, r the like. Amino
acids such as tyrosine, cysteine, lysine, glutamic r aspartic
acid, or the like, can be introduced at the C- r N-terminus
of the peptide or oligopeptide. M dification at the C

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terminus in some cases may alter binding characteristics of the peptide. In additi n, the peptide or ligopeptide sequences can diff r fr m the natural sequence by being modified by terminal-NH_2 acylation, e.g., by alkanoyl (C_1-C_{20})

5   or thioglycolyl acetylation, terminal-carboxyl amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a

10   wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example,

15   Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), supra.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression

20   vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally.in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York

25   (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length

30   contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can

35   then be provided with appropriate linkers and ligated into expressi n v ct rs c mmonly available in the art, and th vect rs used t transform suitable h sts t pr duce the desired fusi n protein. A numb r of such vectors and suitable

25

h st systems are now availabl . For expressi n f the fusion
pr teins, the coding s quenc will be pr vided with perably
linked start and stop codons, promoter and terminator regions
and usually a replication system to provide an expression
5   vector for expression in the desired cellular host. For
example, promoter sequences compatible with bacterial hosts
are provided in plasmids containing convenient restriction
sites for insertion of the desired coding sequence. The
resulting expression vectors are transformed into suitable
10   bacterial hosts. Of course, yeast or mammalian cell hosts may
also be used, employing suitable vectors and control
sequences.

        The peptides of the present invention and
pharmaceutical and vaccine compositions thereof are useful for
15   administration to mammals, particularly humans, to treat
and/or prevent viral infection and cancer. Examples of
diseases which can be treated using the immunogenic peptides
of the invention include prostate cancer, hepatitis B,
hepatitis C, AIDS, renal carcinoma, cervical carcinoma,
20   lymphoma, CMV and condlyloma acuminatum.

        For pharmaceutical compositions, the immunogenic
peptides of the invention are administered to an individual
already suffering from cancer or infected with the virus of
interest. Those in the incubation phase or the acute phase of
25   infection can be treated with the immunogenic peptides
separately or in conjunction with other treatments, as
appropriate. In therapeutic applications, compositions are
administered to a patient in an amount sufficient to elicit an
effective CTL response to the virus or tumor antigen and to
30   cure or at least partially arrest symptoms and/or
complications. An amount adequate to accomplish this is
defined as "therapeutically effective dose." Amounts
effective for this use will depend on, e.g., the peptide
composition, the manner of administration, the stage and
35   severity of the disease being treated, the weight and gen ral
state f health f th patient, and the judgment of the
prescribing physician, but generally range f r the initial
immunizati n (that is for therap utic r pr phylactic

administration) from about 1.0 μg to about 5000 μg of peptide
for a 70 kg patient, foll wed by boosting dosages f from
ab ut 1.0 μg t about 1000 μg of peptide pursuant to a
boosting regimen over weeks to months depending upon the

5   patient's response and condition by measuring specific CTL
activity in the patient's blood. It must be kept in mind that
the peptides and compositions of the present invention may
generally be employed in serious disease states, that is,
life-threatening or potentially life threatening situations.

10   In such cases, in view of the minimization of extraneous
substances and the relative nontoxic nature of the peptides,
it is possible and may be felt desirable by the treating
physician to administer substantial excesses of these peptide
compositions.

15   For therapeutic use, administration should begin at
the first sign of viral infection or the detection or surgical
removal of tumors or shortly after diagnosis in the case of
acute infection. This is followed by boosting doses until at
least symptoms are substantially abated and for a period

20   thereafter. In chronic infection, loading doses followed by
boosting doses may be required.

            Treatment of an infected individual with the
compositions of the invention may hasten resolution of the
infection in acutely infected individuals. For those

25   individuals susceptible (or predisposed) to developing chronic
infection the compositions are particularly useful in methods
for preventing the evolution from acute to chronic infection.
Where the susceptible individuals are identified prior to or
during infection, for instance, as described herein, the

30   composition can be targeted to them, minimizing need for
administration to a larger population.

            The peptide compositions can also be used for the
treatment of chronic infection and to stimulate the immune
system to eliminate virus-infected cells in carriers. It is

35   important to provide an amount of immuno-potentiating peptide
in a formulati n and mode f administration sufficient t
effectively stimulate a cytotoxic T cell response. Thus, f r
treatment f chronic infection, a representative dose is in

the range f ab ut 1.0 μg to about 5000 μg, preferably about 5
μg to 1000 μg for a 70 kg patient per dose. Immunizing d ses
followed by boosting doses at established intervals, e.g.,
from one to four weeks, may be required, possibly for a
prolonged period of time to effectively immunize an
individual. In the case of chronic infection, administration
should continue until at least clinical symptoms or laboratory
tests indicate that the viral infection has been eliminated or
substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic
treatment are intended for parenteral, topical, oral or local
administration. Preferably, the pharmaceutical compositions
are administered parenterally, e.g., intravenously,
subcutaneously, intradermally, or intramuscularly. Thus, the
invention provides compositions for parenteral administration
which comprise a solution of the immunogenic peptides
dissolved or suspended in an acceptable carrier, preferably an
aqueous carrier. A variety of aqueous carriers may be used,
e.g., water, buffered water, 0.9% saline, 0.3% glycine,
hyaluronic acid and the like. These compositions may be
sterilized by conventional, well known sterilization
techniques, or may be sterile filtered. The resulting aqueous
solutions may be packaged for use as is, or lyophilized, the
lyophilized preparation being combined with a sterile solution
prior to administration. The compositions may contain
pharmaceutically acceptable auxiliary substances as required
to approximate physiological conditions, such as pH adjusting
and buffering agents, tonicity adjusting agents, wetting
agents and the like, for example, sodium acetate, sodium
lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the
invention in the pharmaceutical formulations can vary widely,
i.e., from less than about 0.1%, usually at or at least about
2% to as much as 20% to 50% or more by weight, and will be
select d primarily by fluid v lumes, visc sities, etc., in
acc rdance with the particular mode of administrati n
selected.

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The peptides of the inventi n may als b
administered via lipos mes, which serve to target the peptid s
to a particular tissue, such as lymphoid tissue, or targeted
selectively to infected cells, as well as increase the half-
life of the peptide composition. Liposomes include emulsions,
foams, micelles, insoluble monolayers, liquid crystals,
phospholipid dispersions, lamellar layers and the like. In
these preparations the peptide to be delivered is incorporated
as part of a liposome, alone or in conjunction with a molecule
which binds to, e.g., a receptor prevalent among lymphoid
cells, such as monoclonal antibodies which bind to the CD45
antigen, or with other therapeutic or immunogenic
compositions. Thus, liposomes filled with a desired peptide
of the invention can be directed to the site of lymphoid
cells, where the liposomes then deliver the selected
therapeutic/immunogenic peptide compositions. Liposomes for
use in the invention are formed from standard vesicle-forming
lipids, which generally include neutral and negatively charged
phospholipids and a sterol, such as cholesterol. The
selection of lipids is generally guided by consideration of,
e.g., liposome size, acid lability and stability of the
liposomes in the blood stream. A variety of methods are
available for preparing liposomes, as described in, e.g.,
Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S.
Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369,
incorporated herein by reference.

     For targeting to the immune cells, a ligand to be
incorporated into the liposome can include, e.g., antibodies
or fragments thereof specific for cell surface determinants of
the desired immune system cells. A liposome suspension
containing a peptide may be administered intravenously,
locally, topically, etc. in a dose which varies according to,
inter alia, the manner of administration, the peptide being
delivered, and the stage of the disease being treated.

     F r s lid compositions, c nventi nal n nt xic solid
carriers may be used which include, for example,
pharmaceutical grades of mannit 1, lactose, starch, magnesium
stearat , s dium saccharin, talcum, c llulose, glucos ,

sucrose, magnesium carbonate, and the like. F r ral
administrati n, a pharmaceutically acceptable nont xic
compositi n is formed by incorporating any of the normally
employed excipients, such as those carriers previously listed,
and generally 10-95% of active ingredient, that is, one or
more peptides of the invention, and more preferably at a
concentration of 25%-75%.

For aerosol administration, the immunogenic peptides
are preferably supplied in finely divided form along with a
surfactant and propellant. Typical percentages of peptides
are 0.01%-20% by weight, preferably 1%-10%. The surfactant
must, of course, be nontoxic, and preferably soluble in the
propellant. Representative of such agents are the esters or
partial esters of fatty acids containing from 6 to 22 carbon
atoms, such as caproic, octanoic, lauric, palmitic, stearic,
linoleic, linolenic, olesteric and oleic acids with an
aliphatic polyhydric alcohol or its cyclic anhydride. Mixed
esters, such as mixed or natural glycerides may be employed.
The surfactant may constitute 0.1%-20% by weight of the
composition, preferably 0.25-5%. The balance of the
composition is ordinarily propellant. A carrier can also be
included, as desired, as with, e.g., lecithin for intranasal
delivery.

In another aspect the present invention is directed
to vaccines which contain as an active ingredient an
immunogenically effective amount of an immunogenic peptide as
described herein. The peptide(s) may be introduced into a
host, including humans, linked to its own carrier or as a
homopolymer or heteropolymer of active peptide units. Such a
polymer has the advantage of increased immunological reaction
and, where different peptides are used to make up the polymer,
the additional ability to induce antibodies and/or CTLs that
react with different antigenic determinants of the virus or
tumor cells. Useful carriers are well known in the art, and
include, e.g., thyroglobulin, albumins such as bovine serum
albumin, tetanus tox id, p lyamin acids such as
poly(lysine:glutamic acid), hepatitis B virus c re pr tein,
hepatitis B virus recombinant vaccine and the like. The

vaccines can also contain a physiologically t lerable
(acc ptable) dilu nt such as water, ph sphate buffered saline,
or saline, and further typically include an adjuvant.
Adjuvants such as incomplete Freund's adjuvant, aluminum
phosphate, aluminum hydroxide, or alum are materials well
known in the art. And, as mentioned above, CTL responses can
be primed by conjugating peptides of the invention to lipids,
such as P_3CSS. Upon immunization with a peptide composition
as described herein, via injection, aerosol, oral, transdermal
or other route, the immune system of the host responds to the
vaccine by producing large amounts of CTLs specific for the
desired antigen, and the host becomes at least partially
immune to later infection, or resistant to developing chronic
infection.

Vaccine compositions containing the peptides of the
invention are administered to a patient susceptible to or
otherwise at risk of viral infection or cancer to elicit an
immune response against the antigen and thus enhance the
patient's own immune response capabilities. Such an amount is
defined to be an "immunogenically effective dose." In this
use, the precise amounts again depend on the patient's state
of health and weight, the mode of administration, the nature
of the formulation, etc., but generally range from about 1.0
μg to about 5000 μg per 70 kilogram patient, more commonly
from about 10 μg to about 500 μg mg per 70 kg of body weight.

In some instances it may be desirable to combine the
peptide vaccines of the invention with vaccines which induce
neutralizing antibody responses to the virus of interest,
particularly to viral envelope antigens.

For therapeutic or immunization purposes, the
peptides of the invention can also be expressed by attenuated
viral hosts, such as vaccinia or fowlpox. This approach
involves the use of vaccinia virus as a vector to express
nucleotide sequences that encode the peptides of the
invention. Upon introduction into an acutely or chronically
infect d h st r into a non-infected h st, the rec mbinant
vaccinia virus expresses the immunogenic peptide, and thereby
elicits a host CTL response. Vaccinia vect rs and methods

useful in immunizati n protoc ls are described in, e.g.,U.S.
Patent No. 4,722,848, incorp rated herein by referenc .
An ther vector is BCG (Bacille Calmette Guerin). BCG v ct rs
are described in Stover et al. (<u>Nature</u> 351:456-460 (1991))
5   which is incorporated herein by reference. A wide variety of
other vectors useful for therapeutic administration or
immunization of the peptides of the invention, e.g.,
<u>Salmonella</u> <u>typhi</u> vectors and the like, will be apparent to
those skilled in the art from the description herein.
10   Antigenic peptides may be used to elicit CTL <u>ex vivo</u>,
as well. The resulting CTL, can be used to treat chronic
infections (viral or bacterial) or tumors in patients that do
not respond to other conventional forms of therapy, or will
not respond to a peptide vaccine approach of therapy. <u>Ex vivo</u>
15   CTL responses to a particular pathogen (infectious agent or
tumor antigen) are induced by incubating in tissue culture the
patient's CTL precursor cells (CTLp) together with a source of
antigen-presenting cells (APC) and the appropriate immunogenic
peptide. After an appropriate incubation time (typically 1-4
20   weeks), in which the CTLp are activated and mature and expand
into effector CTL, the cells are infused back into the
patient, where they will destroy their specific target cell
(an infected cell or a tumor cell). In order to optimize the
<u>in vitro</u> conditions for the generation of specific cytotoxic T
25   cells, the culture of stimulator cells is maintained in an
appropriate serum-free medium.
           Prior to incubation of the stimulator cells with the
cells to be activated, e.g., precursor CD8+ cells, an amount
of antigenic peptide is added to the stimulator cell culture,
30   of sufficient quantity to become loaded onto the human Class I
molecules to be expressed on the surface of the stimulator
cells. In the present invention, a sufficient amount of
peptide is an amount that will allow about 200, and preferably
200 or more, human Class I MHC molecules loaded with peptide
35   to be expressed on the surface of each stimulator cell.
Preferably, the stimulat r cells are incubat d with >20μg/ml
peptid .

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Resting or precursor CD8+ cells are then incubated in
culture with th appropriate stimulator cells for a time
p ri d sufficient to activate the CD8+ cells. Preferably, the
CD8+ cells are activated in an antigen-specific manner. The
5   ratio of resting or precursor CD8+ (effector) cells to
stimulator cells may vary from individual to individual and
may further depend upon variables such as the amenability of
an individual's lymphocytes to culturing conditions and the
nature and severity of the disease condition or other
10   condition for which the within-described treatment modality is
used. Preferably, however, the lymphocyte:stimulator cell
ratio is in the range of about 30:1 to 300:1. The
effector/stimulator culture may be maintained for as long a
time as is necessary to stimulate a therapeutically useable or
15   effective number of CD8+ cells.

The induction of CTL *in vitro* requires the specific
recognition of peptides that are bound to allele specific MHC
class I molecules on APC. The number of specific MHC/peptide
complexes per APC is crucial for the stimulation of CTL,
20   particularly in primary immune responses. While small amounts
of peptide/MHC complexes per cell are sufficient to render a
cell susceptible to lysis by CTL, or to stimulate a secondary
CTL response, the successful activation of a CTL precursor
(pCTL) during primary response requires a significantly higher
25   number of MHC/peptide complexes. Peptide loading of empty
major histocompatability complex molecules on cells allows the
induction of primary cytotoxic T lymphocyte responses. Peptide
loading of empty major histocompatability complex molecules on
cells enables the induction of primary cytotoxic T lymphocyte
30   responses.

Since mutant cell lines do not exist for every human
MHC allele, it is advantageous to use a technique to remove
endogenous MHC-associated peptides from the surface of APC,
followed by loading the resulting empty MHC molecules with the
35   immunogenic peptides of interest. The use of non-transformed
(non-tum rigenic), n n-infected cells, and preferably,
aut l g us cells of patients as APC is desirable for the
design of CTL induction protocols directed towards dev lopment

of *ex vivo* CTL therapi s. This application discloses meth ds
for stripping the endogen us MHC-ass ciated peptides fr m the
surface of APC foll wed by the loading of d sired peptides.

A stable MHC class I molecule is a trimeric complex
formed of the following elements: 1) a peptide usually of 8 –
10 residues, 2) a transmembrane heavy polymorphic protein
chain which bears the peptide-binding site in its $\alpha1$ and $\alpha2$
domains, and 3) a non-covalently associated non-polymorphic
light chain, $\beta_2$microglobulin. Removing the bound peptides
and/or dissociating the $\beta_2$microglobulin from the complex
renders the MHC class I molecules nonfunctional and unstable,
resulting in rapid degradation. All MHC class I molecules
isolated from PBMCs have endogenous peptides bound to them.
Therefore, the first step is to remove all endogenous peptides
bound to MHC class I molecules on the APC without causing
their degradation before exogenous peptides can be added to
them.

Two possible ways to free up MHC class I molecules of
bound peptides include lowering the culture temperature from
37°C to 26°C overnight to destablize $\beta_2$microglobulin and
stripping the endogenous peptides from the cell using a mild
acid treatement. The methods release previously bound
peptides into the extracellular environment allowing new
exogenous peptides to bind to the empty class I molecules.
The cold-temperature incubation method enables exogenous
peptides to bind efficiently to the MHC complex, but requires
an overnight incubation at 26°C which may slow the cell's
metabolic rate. It is also likely that cells not actively
synthesizing MHC molecules (e.g., resting PBMC) would not
produce high amounts of empty surface MHC molecules by the
cold temperature procedure.

Harsh acid stripping involves extraction of the
peptides with trifluoroacetic acid, pH 2, or acid denaturation
of the immunoaffinity purified class I-peptide complexes.
These methods are not feasible for CTL inducti n, since it is
imp rtant to remove the endogen us peptides while preserving
APC viability and an optimal metabolic stat which is critical
f r antigen presentation. Mild acid solutions of pH 3 such as

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glycine or citrate-phosphate buffers have been used to
identify endogen us peptides and to identify tum r ass ciated
T cell epitopes. The tr atment is specially effective, in
that only the MHC class I molecules are destabilized (and

5   associated peptides released), while other surface antigens
remain intact, including MHC class II molecules. Most
importantly, treatment of cells with the mild acid solutions
do not affect the cell's viability or metabolic state. The
mild acid treatment is rapid since the stripping of the

10   endogenous peptides occurs in two minutes at 4°C and the APC
is ready to perform its function after the appropriate
peptides are loaded. The technique is utilized herein to make
peptide-specific APCs for the generation of primary
antigen-specific CTL. The resulting APC are efficient in

15   inducing peptide-specific CD8+ CTL.

        Activated CD8+ cells may be effectively separated
from the stimulator cells using one of a variety of known
methods. For example, monoclonal antibodies specific for the
stimulator cells, for the peptides loaded onto the stimulator

20   cells, or for the CD8+ cells (or a segment thereof) may be
utilized to bind their appropriate complementary ligand.
Antibody-tagged molecules may then be extracted from the
stimulator-effector cell admixture via appropriate means,
e.g., via well-known immunoprecipitation or immunoassay

25   methods.

        Effective, cytotoxic amounts of the activated CD8+
cells can vary between *in vitro* and *in vivo* uses, as well as
with the amount and type of cells that are the ultimate target
of these killer cells. The amount will also vary depending on

30   the condition of the patient and should be determined via
consideration of all appropriate factors by the practitioner.
Preferably, however, about 1×10^6 to about 1×10^{12}, more
preferably about 1×10^8 to about 1×10^{11}, and even more
preferably, about 1×10^9 to about 1×10^{10} activated CD8+

35   cells are utilized for adult humans, compared to about 5×10^6
- 5×10^7 cells used in mice.

        Preferably, as discussed above, the activated CD8+
cells ar harv sted fr m the cell culture prior to

35

administrati n of the CD8+ cells to the individual being
treated. It is imp rtant to note, however, that unlike ther
present and proposed treatment modalities, the present method
uses a cell culture system that is not tumorigenic.

5   Therefore, if complete separation of stimulator cells and
activated CD8+ cells is not achieved, there is no inherent
danger known to be associated with the administration of a
small number of stimulator cells, whereas administration of
mammalian tumor-promoting cells may be extremely hazardous.

10   Methods of re-introducing cellular components are
known in the art and include procedures such as those
exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and
U.S. Patent No. 4,690,915 to Rosenberg. For example,
administration of activated CD8+ cells via intravenous
15 infusion is appropriate.

            The immunogenic peptides of this invention may also
be used to make monoclonal antibodies. Such antibodies may be
useful as potential diagnostic or therapeutic agents.

            The peptides may also find use as diagnostic
20 reagents. For example, a peptide of the invention may be used
to determine the susceptibility of a particular individual to
a treatment regimen which employs the peptide or related
peptides, and thus may be helpful in modifying an existing
treatment protocol or in determining a prognosis for an
25 affected individual. In addition, the peptides may also be
used to predict which individuals will be at substantial risk
for developing chronic infection.

            The following examples are offered by way of
illustration, not by way of limitation.
30

Example 1
Class I antigen isolation

            A flow diagram of an HLA-A antigen purification
scheme is presented in Figure 1. Briefly, the cells bearing
35 the appropriate allele were grown in large batches (6-8 liters
yielding ⁻5 x 10⁹ cells), harvested by centrifugation and
washed. All cell lines were maintained in RPMI 1640 media
(Sigma) suppl mented with 10% fetal bovine serum (FBS) and

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antibiotics. For large-scale cultures, cells were grown in
roller b ttle culture in RPMI 1640 with 10% FBS or with 10%
horse serum and antibiotics. Cells were harvested by
centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259
5   rotor and washed three times with phosphate-buffered saline
(PBS)(0.01 M PO_4, 0.154 M NaCl, pH 7.2).

Cells were pelleted and stored at -70°C or treated
with detergent lysing solution to prepare detergent lysates.
Cell lysates were prepared by the addition of stock detergent
10   solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci.
Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0]
to the cell pellets (previously counted) at a ratio of 50-100
x 10^6 cells per ml detergent solution. A cocktail of protease
inhibitors was added to the premeasured volume of stock
15   detergent solution immediately prior to the addition to the
cell pellet. Addition of the protease inhibitor cocktail
produced final concentrations of the following:
phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5
µg/ml; leupeptin, 10 µg/ml; pepstatin, 10 µg/ml;
20   iodoacetamide, 100 µM; and EDTA, 3 ng/ml. Cell lysis was
allowed to proceed at 4°C for 1 hour with periodic mixing.
Routinely 5-10 x 10^9 cells were lysed in 50-100 ml of
detergent solution. The lysate was clarified by
centrifugation at 15,000 x g for 30 minutes at 4°C and
25   subsequent passage of the supernatant fraction through a 0.2 µ
filter unit (Nalgene).

The HLA-A antigen purification was achieved using
affinity columns prepared with mAb-conjugated Sepharose beads.
For antibody production, cells were grown in RPMI with 10% FBS
30   in large tissue culture flasks (Corning 25160-225).
Antibodies were purified from clarified tissue culture medium
by ammonium sulfate fractionation followed by affinity
chromatography on protein-A-Sepharose (Sigma). Briefly,
saturated ammonium sulfate was added slowly with stirring to
35   the tissue culture supernatant to 45% (volume to v lume)
vernight at 4°C to precipitate the immunogl bulins. The
precipitated pr teins were harvested by centrifugation at
10,000 x g for 30 minutes. The precipitate was then dissolved

in a minimum volum of PBS and transf rred t dialysis tubing
(Spectr /P r 2, Mol. wt. cut ff 12,000-14,000, Spectum Medical
Ind.). Dialysis was against PBS (≥20 times the protein
solution volume) with 4-6 changes of dialysis buffer over a
24-48 hour period at 4°C. The dialyzed protein solution was
clarified by centrifugation (10,000 x g for 30 minutes) and
the pH of the solution adjusted to pH 8.0 with 1N NaOH.
Protein-A-Sepharose (Sigma) was hydrated according to the
manufacturer's instructions, and a protein-A-Sepharose column
was prepared. A column of 10 ml bed volume typically binds
50-100 mg of mouse IgG.

        The protein sample was loaded onto the protein-A-
Sepharose column using a peristaltic pump for large loading
volumes or by gravity for smaller volumes (<100 ml). The
column was washed with several, volumes of PBS, and the eluate
was monitored at A280 in a spectrophotometer until base line
was reached. The bound antibody was eluted using 0.1 M citric
acid at suitable pH (adjusted to the appropriate pH with 1N
NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was
used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base
was used to neutralize the eluate. Fractions containing the
antibody (monitored by A280) were pooled, dialyzed against PBS
and further concentrated using an Amicon Stirred Cell system
(Amicon Model 8050 with YM30 membrane). The anti-A2 mAb,
BB7.2, and the anti-A3 mAb, GAPA3, are particularly useful for
affinity purification.

        The HLA-A antigen was purified using affinity columns
prepared with mAb-conjugated Sepharose beads. The affinity
columns were prepared by incubating protein-A-Sepharose beads
(Sigma) with affinity-purified mAb as described above. Five
to 10 mg of mAb per ml of bead is the preferred ratio. The
mAb bound beads were washed with borate buffer (borate buffer:
100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the
washes show A280 at based line. Dimethyl pimelimidate (20 mM)
in 200 mM triethanolamine was added to c valently cr sslink
the bound mAb to the pr tein-A-Sephar se (Schneider et al., J.
Biol. Chem. 257:10766 (1982). After incubation f r 45 minutes
at r om temperature on a rotator, the excess crosslinking

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reag nt was removed by washing the beads twice with 10-20 ml
of 20 mM ethan lamine, pH 8.2. Between each wash the slurry
was placed n a rotat r for 5 minutes at ro m temperature.
The beads were washed with borate buffer and with PBS plus
0.02% sodium azide.

   The cell lysate (5-10 x 10^9 cell equivalents) was
then slowly passed over a 5-10 ml affinity column (flow rate
of 0.1-0.25 ml per minute) to allow the binding of the antigen
to the immobilized antibody. After the lysate was allowed to
pass through the column, the column was washed sequentially
with 20 column volumes of detergent stock solution plus 0.1%
sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM
Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0.
The HLA-A antigen bound to the mAb was eluted with a basic
buffer solution (50 mM diethylamine in water). As an
alternative, acid solutions such as 0.15-0.25 M acetic acid
were also used to elute the bound antigen. An aliquot of the
eluate (1/50) was removed for protein quantification using
either a colorimetric assay (BCA assay, Pierce) or by SDS-
PAGE, or both. SDS-PAGE analysis was performed as described
by Laemmli (Laemmli, U.K., _Nature_ 227:680 (1970)) using known
amounts of bovine serum albumin (Sigma) as a protein standard.

   Allele specific antibodies were used to purify the
specific MHC molecule. In the case of HLA-A2 and HLA-A3 mAbs
BB7.2 and GAPA3 were used respectively. An example of SDS
PAGE analysis of purified HLA-A3.2 molecules is shown in
Figure 2.

   Figure 2 shows SDS-PAGE (12.5%) analysis of affinity
purified HLA-A3.2 from the cell line EHM. An affinity column
(10 ml) was prepared with protein A-sepharose beads coupled to
the monoclonal antibody GAPA3 which is specific for HLA-A3. A
detergent lysate of $5x10^9$ cells was passaged over the column
and the column was washed extensively. The bound HLA-A3.2
molecules were eluted from the column with 0.15M acetic acid,
50 ml. One ml of the eluate was removed and lyophilized to
c ncentrate the sampl . Th sample was taken up to 50 µl with
Leammli sample buffer and 20 µl were loaded in lane 2. Lane 1
contained molecular weight standards: Myosin, 230 kD; ß-

galact sidase, 116kD; ph sphorylase B, 97.4kD; bovine serum
albumin, 66.2kD; valbumin, 45kD; carb nic anhydrase, 31kD;
soybean trypsin inhibitor, 21.5kD; and lysozyme, 14.4kD.
Standard concentrations of bovine serum albumin were run in
5   lanes 8, 10 μg, 9, 3 μg, and 10, 1μg to aid in the estimation
of protein yield. For this particular HLA-A3.2 preparation,
the estimated yield was approximately 112 μg.

For HLA-A11, A24.1 and A1, an alternative protocol
was used whereby anti-HLA-B and C monoclonal antibodies were
10   used to deplete HLA-B and C molecules. The remaining HLA-A
molecules were subsequently purified using the W6/32 mAb as
described below.

Based on the density of class I expression as
indicated by the results of immunofluorescent staining
15   analysis, it is anticipated that average yields of class I
antigen isolated from the EBV B cell lines will range from
800-1200 μg per 10^{10} cell equivalents.

Example 2
20   ## An alternative class I purification protocol
HLA-A2.1 molecules were isolated using the mAb
B1.23.2 which detects an epitope expressed by HLA-B and C
allele molecules, but not by HLA-A antigens. The mAb, W6/32,
detects all human class I molecules, including HLA-A, B and C.
25   As mentioned above, these mAbs react well with the B cell
lines serving as sources of HLA-A antigens. The B1.23.2 mAb
reacts with the various human B cell lines, but fails to react
with a mouse cell line that expresses a transfected HLA-A2.1
protein or a chimeric A2.1 mouse K^b molecule. It does react
30   with the human cell line, CIR (Alexander, J., et al.,
Immunogenetics, 29, 380 [1989]), that lacks expression of
HLA-A and B molecules, but expresses low levels of HLA-C
molecules. This pattern of reactivity illustrates how the
B1.23.2 mAb can be used to deplete the B cell lysates of HLA-B
35   and C molecules.

Affinity c lumns were prepared using the affinity-
purified B1.23.2 and W6/32 mAbs, respectively, as described
ab ve. The pr cedures for the preparation of the affinity

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columns are essentially identical to the procedur s described
f r the preparation of the allele-sp cific mAb c lumns
described ab ve. The B1.23.2 mAb affinity column was used to
deplete the detergent lysates of HLA-B and C molecules using
5   the protocol as described above. The cell lysate depleted of
HLA-B and C was then passed over a W6/32 mAb affinity column.
The MHC molecule that was eluted from this second passage was
the A allele product.

        This alternative affinity purification is useful for
10   the purification of any HLA-A allele product, and does not
rely on the need for allele-specific mAbs. In addition, it
could also be used to isolate any class I molecule type from
transfected cell lines.

15   Example 3
        Isolation and sequencing of naturally processed peptides
        For the HLA-A preparations derived from the base (50
mM diethylamine) elution protocol, the eluate was immediately
neutralized with 1 N acetic acid to pH 7.0-7.5. The
20   neutralized eluate was concentrated to a volume of 1-2 ml in
an Amicon stirred cell [Model 8050, with YM3 membranes
(Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was
added to the concentrator to remove the non-volatile salts,
and the sample was concentrated to approximately 1 ml. A
25   small sample (1/50) was removed for protein quantitation as
described above. The remainder was recovered into a 15 ml
polypropylene conical centrifuge tube (Falcon, 2097) (Becton
Dickinson). Glacial acetic acid was added to obtain a final
concentration of 10% acetic acid. The acidified sample was
30   placed in a boiling water bath for 5 minutes to allow for the
dissociation of the bound peptides. The sample was cooled on
ice, returned to the concentrator and the filtrate was
collected. Additional aliquots of 10% acetic acid (1-2 ml)
were added to the concentrator, and this filtrate was pooled
35   with the original filtrate. Finally, 1-2 ml of distilled
water was add d t the c ncentrat r, and this filtrate was
p led as well.

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The ret ntate c ntains the bulk of the HLA-A heavy
chain and β_2-micr globulin, while the filtrate contains the
naturally processed bound peptides and other components with
molecular weights less than about 3000. The pooled filtrate
5   material was lyophilized in order to concentrate the peptide
fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography)
separation of the peptide fractions, the lyophilized sample
was dissolved in 50 μl of distilled water, or into 0.1%
10   trifluoracetic acid (TFA) (Applied Biosystems) in water and
injected to a C18 reverse-phase narrow bore column (Beckman
C18 Ultrasphere, 10 x 250 mm), using a gradient system
described by Stone and Williams (Stone, K.L. and Williams
K.R., in, Macromolecular Sequencing and Synthesis; Selected
15   Methods and Applications, A.R. Liss, New York, 1988, pp. 7-24.
Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B
was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The
flow rate was 0.250 ml/minute with the following gradient: 0-
60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-
20   98% B. The Gilson narrow bore HPLC configuration is
particularly useful for this purpose, although other
configurations work equally well.

A large number of peaks were detected by absorbance
at 214 nm, many of which appear to be of low abundance
25   (Fig. 3). Whether a given peak represents a single peptide or
a peptide mixture was not determined. Pooled fractions were
then sequenced to determine motifs specific for each allele as
described below.

Pooled peptide fractions, prepared as described above
30   were analyzed by automated Edman sequencing using the Applied
Biosystems Model 477A automated sequencer. The sequencing
method is based on the technique developed by Pehr Edman in
the 1950s for the sequential degradation of proteins and
peptides to determine the sequence of the constituent amino
35   acids.

The pr tein or peptide t b sequenced was held by a
12-mm diameter p rous glass fiber filter disk in a heated,
argon-purged reaction chamber. The filter was generally pr -

treated with Bi Brene Plus™ and then cycled through one or
m re r petitions f the Edman reaction to reduce c ntaminants
and improve the efficiency of subsequent sample sequencing.
Following the pre-treatment of the filter, a solution of the
sample protein or peptide (10 pmol-5 nmol range) was loaded
onto the glass filter and dried. Thus, the sample was left
embedded in the film of the pre-treated disk. Covalent
attachment of the sample to the filter was usually not
necessary because the Edman chemistry utilized relatively
apolar solvents, in which proteins and peptides are poorly
soluble.

Briefly, the Edman degradation reaction has three
steps: coupling, cleavage, and conversion. In coupling step,
phenylisothiocyanate (PITC) is added. The PITC reacts
quantitatively with the free amino-terminal amino acid of the
protein to form the phenylthiocarbamyl-protein in a basic
environment. After a period of time for the coupling step,
the excess chemicals are extracted and the highly volatile
organic acid, trifluoroacetic acid, TFA, is used to cleave the
PITC-coupled amino acid residue from the amino terminus of the
protein yielding the anilinothiazolinone (ATZ) derivative of
the amino acid. The remaining protein/peptide is left with a
new amino terminus and is ready for the next Edman cycle. The
ATZ amino acid is extracted and transferred to a conversion
flask, where upon addition of 25% TFA in water, the ATZ amino
acid is converted to the more stable phenylthiohydantoin (PTH)
amino acid that can be identified and quantified following
automatic injection into the Model 120 PTH Analyzer which uses
a microbore C-18 reverse-phase HPLC column for the analysis.

In the present procedures, peptide mixtures were
loaded onto the glass filters. Thus, a single amino acid
sequence usually does not result. Rather, mixtures of amino
acids in different yield are found. When the particular
residue is conserved among the peptides being sequenced,
increased yield for that amino acid is observed.

Example 4

Definition f an A3.2 specific motif

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There is some ambiguity in th internati nal
nomenclature f A3 alleles. The A3.2 allele herein is
expressed by cell lines EHM, HO301, and GM3107. This
particular subtype is currently referred to as the 3.2 allele
5   (Yang, in _Immunobiology of HLA_, Vol. 1, Dupont ed., Springer-
Verlag, New York pp. 43-44 and 54-55, 1989), or the product of
the A*0301 gene (its sequence corresponds to the one published
by Strachan, et al., _EMBO J._, 3:887 (1984), and has been
verified by direct cloning and sequencing of the A3 gene found
10   in EHM cell line. The HLA-A3.2 encoded by the A*0301 gene
referred to in this document is the commonly expressed HLA-A3
allelic form.

In one case using MAT cells, pooled peptide fractions
prepared as described in Example 3 above were obtained from
15   HLA-A3.2 homozygous cell lines, for example, CM3107. The
pooled fractions were HPLC fractions corresponding to 7% to
19% CH_3CN. For this class I molecule, this region of the
chromatogram was most abundant in peptides. Data from
independent experiments were averaged as described below.
20   The amino acid sequence analyses from four
independent experiments were analyzed and the results are
shown in Table 5. For each position except the first, the
data were analyzed by modifying the method described by Falk
et al. to allow for comparison of experiments from different
25   HLA types. This modified procedure yielded quantitative yet
standardized values while allowing the averaging of data from
different experiments involving the same HLA type.

The raw sequenator data was converted to a simple
matrix of 10 rows (each representing one Edman degradation
30   cycle) and 16 columns (each representing one of the twenty
amino acids; W, C, R and H were eliminated for technical
reasons. The data corresponding to the first row (first
cycle) was not considered further because, this cycle is
usually heavily contaminated by free amino acids.). The
35   values of each row were summed to yield a t tal pmol s value
f r that particular cycle. F r ach r w, values for each
amino acid were then divided by the c rr sp nding t tal yield
value, to determine what fraction of the total signal is

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attributable to each amino acid at each cycle. By doing so,
an "Absolute Fr quency" table was generated. This absolute
frequency table allows corr cti n f r the declining yields of
each cycle.

5   Starting from the absolute frequency table, a
"relative frequency" table was then generated to allow
comparisons among different amino acids. To do so the data
from each column was summed, and then averaged. Then, each
value was divided next by the average column value to obtain
10   relative frequency values. These values quantitate, in a
standardized manner, increases and decreases per cycle, for
each of the different sixteen amino acid types. Tables
generated from data from different experiments can thus be
added together to generate average relative frequency values
15   (and their standard deviations). All standard deviations can
then be averaged, to estimate a standard deviation value
applicable to the samples from each table. Any particular
value exceeding 1.00 by more than two standard deviations is
considered to correspond to a significant increase.

20   The results of the foregoing analysis for HLA-A3.2
were as follows: at position 2, a 2.2-fold increase in valine
(V) with lesser increases (1.5-1.7) for structurally similar
residues leucine (L) and methionine (M). At position 3,
tyrosine (Y) and aspartic acid (D) showed increases in
25   frequency. At position 7 isoleucine (I) was increased, and at
position 8 asparagine (N) and glutamine (Q) were increased.
At positions 9 and 10, lysine (K) was increased more than 2-
fold over the expected random yield.

          Cysteine was not modified and thus not detected.
30   PTH-tryptophan coeluted with diphenylurea, and in some
experiments, PTH-arginine coeluted with the major derivative
of PTH-threonine. Therefore, cysteine and tryptophan are not
detectable and arginine is detected only in the absence of
threonine.

35   Previously described MHC structures showed instances
of critically conserved residues at position 2 (or 3) and at
the C terminus (either position 9 or 10). These residues are
referred to as "conserv d" residues. The modified data

analysis f this invention considered th c nserved positi ns
at the N and C terminals.

Thus, the HLA-A3.2 motif sh uld have p sition two
occupied by V, L or M, a length of 9 or 10 amino acids, and a
C-terminal position occupied by K.

TABLE 5
Summary
HLA-A3.2 Allele-Specific Motif

Position	Conserved Residues
1	-
2	V,L,M
3	Y,D
4	-
5	-
6	-
7	I
8	Q,N
9	K
10	K

Example 5

Definition of HLA-A1-specific peptide motifs

HLA-A1 molecules were isolated and their naturally
processed peptides characterized, as described in Example 3
above. In one case using MAT cells, pooled fractions
corresponding to 19% to 50% CH_3CN were used. As in the
preceding example, residues showing at any given position
except the first position, at least a two standard deviation
increase over the random expected yield were identified and
shown in Table 6. On the basis of these data, only Serine (S)
and Threonine (T) were increased at position two. At position
3, aspartic acid (D) and glutamic acid (E) were elevated and
at position 9 and 10 tyrosine (Y) showed a marked increase.
Other increases noted were proline (P) at positi n 4 and
leucine (L) at p siti n 7. Th ref re, th m tifs for HLA-A1
based n thes data would have residues at p sition 2 ccupied
by S or T, a peptide l ngth of 9 or 10 amino acids and a

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C-terminal residue of Y. Alternatively, another motif w uld
c mprise a D r E at positi n 3 together with a C terminal
r sidu of Y.

TABLE 6
Summary
HLA-A1 Allele-Specific Motif

Position	Conserved Residues
1	-
2	S, T
3	D,E
4	P
5	-
6	-
7	L
8	-
9	Y

Example 6
Definition of HLA-A11 allele-specific peptide motifs

HLA-A11 motifs were defined by amino acid sequence
analysis of pooled HPLC fractions, in one case corresponding
to 7% to 45% CH_3CN of fractionated peptides eluted from
HLA-A11 molecules purified from the cell line BVR. On the
basis of the data presented in Table 7, a motif for A11
consists of a conserved residue at position 2 of threonine (T)
or valine (V), a peptide length of 9 or 10 amino acids, and a
C-terminal conserved residue of lysine (K). At position 3
increases in methionine (M) and phenylalanine (F) were also
seen and at position 8 glutamine (Q) was increased.

TABLE 7
Summary
HLA-A11 Allele-Specific Motif

Position	Conserved Residues
1	-
2	T,V
3	M,F
4	-

5	–
6	–
7	–
8	Q
9	K
10	K

Example 7

Definition of HLA-A24.1 Specific Peptide Motifs

HLA-A24.1 allele-specific motifs were defined by amino acid sequence analysis of pooled fractions in one case corresponding to 7% to 19% CH_3CN of HPLC fractionated peptides eluted from HLA-A24.1 molecules purified from the cell line KT3. On the basis of the data presented in Table 8 a motif for HLA-A24.1 consists of a conserved residue at position 2 occupied by tyrosine (Y), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of phenylalanine (F) or leucine (L). Increases were also observed at several other positions: isoleucine (I) and methonine (M) at position 3; aspartic acid (D), glutamic acid (E), glycine (G), lysine (K) and proline (P) at position 4; lysine (K), methonine (M) and asparagine (N) at position 5; valine (V) at position 6; asparagine (N) and valine (V) at position 7; and, alanine (A), glutamic acid (E), lysine (K), glutamine (Q) and serine (S) at position 8.

Table 8
Summary

HLA-A24.1 Allele-Specific Motif

Position	Conserved Residues
1	–
2	Y
3	I,M
4	D,E,G,K,P
5	L,M,N
6	V
7	N,V

8	A,E,K,Q,S
9	F,L
10	F,A

Example 8

Identification of immunogenic peptides

Using the motifs identified above for various MHC class I allele amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of these motifs. Sequences for all of the target antigens were obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs was done using the "FINDPATTERNS" program (Devereux, Haeberli and Smithes (1984). Nucleic Acids Research 12(1); 387-395).

The amino acid sequence or the nucleotide sequence encoding products was obtained from the GenBank database. In the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu), and Melanoma Antigen-1 (MAGE-1), a single sequence exists.

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs were identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr were added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 was derived from 9 viral isolates. Motifs were identified on those regions that had no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates were identified and add d t the peptide list.

Finally, a consensus s quence for HIV type 1 for North American viral isolates (10-12 viruses) was btained

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from the Los Alam s Nati nal Lab rat ry database (May 1991
release) and analyzed in rder to identify m tifs that ar
constant thr ugh ut most viral isolates. Motifs that bear a
small degree of variation (one residue, in 2 forms) were also
5   added to the peptide list.

Several motifs for each allele shown below were used
to screen several antigens. Protein E6 of human papilloma
virus (HPV) type 16 using motifs from all of the alleles
disclosed above are shown (Table 9). Protein E7 of HPV type
10   18 was also searched for motifs from all alleles (Table 9)
Melanoma antigens MAGE 1, 2 and 3 were searched for motifs
from all alleles (Table 10). The antigen PSA was searched for
motifs from all alleles (Table 11). Finally, core and
envelope proteins from hepatitis C virus were also searched
15   (Table 12). In the tables and the description of the motifs,
the conventional symbol letter for each amino acid was used.
The letter "X" represents a wild card character (any amino
acid).

The following motifs were screened in the present
20   search:

For HLA-A1 (A*0101):
1   XSXXXXXXY
2   XSXXXXXXXY
25   3   XTXXXXXXY
4   XTXXXXXXXY
5   XXDXXXXXY
6   XXDXXXXXXY
7   XXEXXXXXY
30   8   XXEXXXXXXY

For HLA-A3.2 (A*0301)
1   XVXXXXXXK
2   XVXXXXXXXK
35   3   XLXXXXXXK
4   XLXXXXXXXK
5   XMXXXXXXK
6   XMXXXXXXXK

50

For HLA-A11 (A*1101):

1   XTXXXXXXK

2   XTXXXXXXXK

3   XVXXXXXXK

4   XVXXXXXXXK

For HLA-A24.1 (A*2401):

1   XYXXXXXXF

2   XYXXXXXXXF

3   XYXXXXXXL

4   XYXXXXXXXL

Peptides with MHC Class I Binding Motifs   Table 9

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AA Position	Sequence	Antigen	HLA molecule
30	IHDIILECVY	HPV16.E6	A1
69	VCDKCLKFY	HPV16.E6	A1
77	YSKISEYRHY	HPV16.E6	A1
80	ISEYRHYCY	HPV16.E6	A1
92	GTTLEQQYNK	HPV16.E6	A11
93	TTLEQQYNK	HPV16.E6	A11
106	LLIRCINCQK	HPV16.E6	A3
2	HGDTPTLHEY	HPV16.E7	A1
16	QPETTDLYCY	HPV16.E7	A1
44	QAEPDRAHY	HPV16.E7	A1
89	IVCPICSQK	HPV16.E7	A3, A11
3	RFEDPTRRPY	HPV18.E6	A1
4	FEDPTRRPY	HPV18.E6	A1
25	LQDIEITCVY	HPV18.E6	A1
41	LTEVFEFAFK	HPV18.E6	A11
72	YSRIRELRHY	HPV18.E6	A1
84	SVYGDTLEK	HPV18.E6	A3, A11
101	LLIRCLRCQK	HPV18.E6	A3
59	HTMLCMCCK	HPV18.E7	A11

Human Papilloma Virus 16 and 18 (E6 and E7 Proteins)

52.

Peptides with MHC Class I Binding Motifs   Table 10

AA Position	Sequence	Antigen	HLA molecule
5	2 SLEQRSLHCK	MAGE 1	A3
	96 SLFRAVITK	MAGE 1	A3
	96 SLFRAVITKK	MAGE 1	A3
	108 DLVGFLLLK	MAGE 1	A3
	128 MLESVIKNYK	MAGE 1	A3
10	128 MLESVIKNY	MAGE 1	A1
	152 QLVFGIDVK	MAGE 1	A3
	161 EADPTGHSY	MAGE 1	A1
	182 LLGDNQIMPK	MAGE 1	A3
	215 WEELSVMEVY	MAGE 1	A1
15	223 VYDGREHSAY	MAGE 1	A1
	238 LLTQDLVQEK	MAGE 1	A3
	239 LTQDLVQEK	MAGE 1	A11
	239 LTQDLVQEKY	MAGE 1	A1
	240 TQDLVQEKY	MAGE 1	A1
20			

Melanoma Antigen MAGE 1

Peptides with MHC Class I Binding M tifs   Table 11

AA Position	Sequence	Antigen	HLA molecule
5	21 IVGGWECEK	PSA	A3, A11
	57 LTAAHCIRNK	PSA	A11
	88 VSHSFPHPLY	PSA	A1
	95 PLYDMSLLK	PSA	A3
	178 DVCAQVHPQK	PSA	A3, A11
10	182 QVHPQKVTK	PSA	A3, A11
	236 PSLYTKVVHY	PSA	A1
	239 YTKVVHYRK	PSA	A11
	241 KVVHYRKWIK	PSA	A3, A11
	242 VVHYRKWIK	PSA	A3, A11
15			

Prostate Specific Antigen (PSA)

54

Peptid s with MHC Class I Binding Motifs   Table 12

AA Positi n	Sequence	Antigen	HLA molecule
2 STNPKPQRK		HCV	A11
14 NTNRRPQDVK		HCV	A11
43 RLGVRATRK		HCV	A3
302 VQDCNCSIY		HCV	A1
556 WMNSTGFTK		HCV	A3
605 LTPRCMVDY		HCV	A1
626 FTIFKIRMY		HCV	A1

5

10

Hepatitis C Virus (Consensus Sequence)

15

55

Example 9

Quantitative HLA class I binding assay

To verify that motif-containing peptide sequences are indeed capable of binding to the appropriate class I

5   molecules, specific binding assays were established. HLA-A3.2 molecules,were purified from GM3107 EBV cells by affinity chromatography using the GAPA3 mAb (anti-A3) to isolate A3.2. Prior to the step, the lysate was depleted of HLA B and C molecules by repeated passages over a B1.23.2 column (this

10   antibody is B,C specific) generally as described in Example 2, above.

As a radiolabeled probe, the peptide 941.12 (KVFPYALINK), containing an A3.2 motif, was used. This peptide contains the anchor residues V_2 and K_{10}, associated

15   with A3.2-specific binders, described above. A Y residue was inserted at position 5 to allow for radiolodination. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

20   A dose range of purified A3.2 was incubated with 10 nM of 941.12 at pH 7.0 and 23°C, in presence of a protease inhibitor cocktail (1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM N a_p-tosyl-L-lysine chloromethyl ketone (TLCK)), in presence of 1 μM purified

25   human β2 microglobulin. After two days, the % bound radioactivity was measured by gel filtration over TSK 2000 columns as previously described for class II peptide binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Gefter, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202,

30   which is incorporated herein by reference. (see, Fig. 4). Good binding (in the 60 to 100% range) was observed for A3.2 concentrations ranging between 35 and 300 nM. 30% binding was observed at 15 nM A3.2.

To minimize A3.2 usage and to increase the

35   sensitivity of the assay, a concentration f 5-10 nM A3.2 was selected f r further assays. In the experiment shown in Fig. 5, 7nM A3.2 and an equivalent c ncentration of radiolabeled 941.12 w r incubated using the conditions describ d above and

in the presence of a dos range of three peptides (HBc 18-27
(924.07), a Prostate Specific Antigen p ptide (939.01), and
HIV nef 73-82 (940.03)). It was f und that peptide 940.03
inhibited strongly, with a 50% inhibitory concentration

5   (IC50%) of 22 nM, while a weaker inhibition was observed with
peptide 939.01 (IC50% 940 nM). Finally, peptide 924.07 did
not show any inhibition up to the 30 μM level. Thus, it is
concluded that peptides 940.03 and 939.01 are high and
intermediate affinity binders, respectively, while peptide

10   924.07 is classified as a low affinity or negative binder.
        Throughout this disclosure, results have been
expressed in terms of IC50's. Given the conditions in which
the assays are run (i.e., limiting MHC and labeled peptide
concentrations), these values approximate K_D values. It

15   should be noted that IC50 values can change, often
dramatically, if the assay conditions are varied, and
depending on the particular reagents used (e.g., Class I
preparation, etc.). For example, excessive concentrations of
MHC will increase the apparent measured IC50 of a given

20   ligand.
        An alternative way of expressing the binding data, to
avoid these uncertainties, is as a relative value to a
reference peptide. The reference peptide is included in every
assay. As a particular assay becomes more, or less,

25   sensitive, the IC50's of the peptides tested may change
somewhat. However, the binding relative to the reference
peptide will not change. For example, in an assay run under
conditions such that the IC50 of the reference peptide
increases 10-fold, all IC50 values will also shift

30   approximately 10-fold. Therefore, to avoid ambiguities, the
assessment of whether a peptide is a good, intermediate, weak,
or negative binder should be based on it's IC50, relative to
the IC50 of the standard peptide.
        If the IC50 of the standard peptide measured in a

35   particular assay is different fr m that rep rted in th table,
then it sh uld be underst od that th threshold values used t
determine good, intermediate, weak, and negative binders
should be modified by a corresponding factor. For example, if

in an A2.1 binding assay, the IC50 of the A2.1 standard
(941.01) were to be measured as 8 nM instead f 5 nM, th n a
peptide ligand would be called a good binder only if it had an
IC50 of less than 80 nM (i.e., 8nM x 0.1), instead of the
usual cut-off value of 50 nM.

        The experimental system herein described can be used
to test binding of large numbers of synthetic peptides to a
variety of different class I specificities. Specific binding
assays can be performed as follows.

HLA-A11-specific assay

        The cell line BVR was used as a source of HLA. The
dependency of the binding on MHC concentration in presence or
absence of β_2M are shown in Fig. 6, while Fig. 7 depicts the
dose dependency of the inhibition by excess unlabeled ligand.
Finally, Fig. 8 shows a Scatchard analysis experiment. Values
of apparent kD of ~6 nM and of 10% active receptor were
obtained, and were remarkable for their similarity to the
values obtained for A2.1 and A3.2. The sequence of the peptide
used as a radiolabeled probe (940-06) is AVDLYHFLK.

HLA-A1-specific assay

        In this case, the EBV cell line Steinlin was used as
a source of purified HLA.. The same protocol previously
applied to purification of other HLA alleles (i.e., depletion
of B, C molecules by a B1.23.2 mAb column, followed by
purification of A molecules by means of a W632 mAb column) was
utilized. On the basis of the pool sequencing data, consensus
peptides were synthesized, directly radiolabeled, and tested
for HLA binding using the standard protocol (1 mM β_2M, 2 days
RT incubation in presence of protease inhibitors). A graph
illustrating the relationship between % binding and μM input
HLA A1 is shown in Fig. 9. From the data, it was concluded
that in analogy with what was observed for HLA A2, 3, and 11,
as little as 30 nM are sufficient to obtain ~10% binding. The
sequence of the peptide used as a radiolabeled pr be (944.02)
is YLEPAIAKY. In the next set f experiments, the specificity
f the assay established was verified by its inhabitability by
exc ss unlabeled peptide. The IC50% was measured (Fig. 10) as

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~20 nM. Further Scatchard analysis (Fig. 11) verified that
the apparent K_D f the interaction c rresponded to 21 nM, with
a % of active r cept r c rresponding t 5.1%.

5   HLA-A24 specific assay

HLA A24 molecules were purified from the KT3 EBV cell
line. In this case, two consensus peptides whose sequences
were based on the pool sequencing data have been synthesized.
Their sequences are: 979-01, AYIDNVYKF and 979.02, AYIDNYNKF.
10 The results of experiments in which the % bound of these two
peptides as a function of input MHC was measured are shown in
Fig. 12. In both cases, 10-15% binding was obtained with as
little as 20-50 nM MHC. Cold inhibition experiments (Fig.
13), limiting MHC concentrations, revealed that the binding
15 was readily inhibitable by excess unlabeled peptide, with an
apparent K_D of 30 and 60 nM, respectively. Further Scatchard
experiments verified values of 136 nM and 28 nM, respectively.
The apparent % of available receptor (active MHC) were 8.3%
and 7.4%, respectively (Fig. 9a and b). On the basis of these
20 data, peptide 979.02 was arbitrarily selected as standard
label indicator for A24 assays. Furthermore, on the basis of
the data herein described, we also conclude that the goal of
establishing an A24-specific binding assay has been
accomplished. In conclusion, specific assays for the five
25 major HLA alleles have been described.

                          Example 10
                   Expansion of HLA A Motifs
Establishing in vitro binding assays allows one to
30 readily quantitate in vitro the binding capacity of various
synthetic peptides to the various alleles of interest (HLA A1,
A2, A3, A11, and A24). This allows verification of the
correctness of the motifs by means of peptides carrying the
various HLA A motifs for their capacity to bind purified HLA
35 molecules. Typically, peptides were synthesized with specific
HLA motifs embedded in a neutral backbone c mposed of only
alanine residues. In some cases, a K residue was als
intr duced within the sequence, with the purpose f incr asing

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solubility. The use of such "neutral" p ly A backb nes, as
applied t the case f class II molecules, has been described
in detail, f r example, by Jardetzky et al. (Jardetzky et al.,
EMBO J. 9(6):1797,1990).

5   For example, in the case of A3.2, a motif has been
defined with a hydrophobic residue in position 2 and a
positive charge (K) in position 9. Thus, to verify that the
presence of these two anchor residues would allow, in the
context of a poly A backbone, for A3.2 binding, the poly A

10   analog with the sequence AMAAAAAAK was synthesized (Table 13).
               Similarly, other peptides carrying other HLA motifs
were also synthesized and tested for HLA binding. It was
found that in all cases, the presence of the specific HLA
motifs was conducive to binding to the relevant HLA allele,

15   with estimated K_D comprised of between 125 and 2.8 nM. In
most cases, the binding was also absolutely specific, in that
no binding was detected to irrelevant alleles. Only two
exceptions to this general rule were observed. Firstly, A3
and A11 peptides crossreacted extensively with each other,

20   perhaps as could have been expected by the fact that the
motifs for these two alleles are remarkably similar. Second,
some A1 peptides crossreacted, albeit with much lower
affinities, on A11 and A3.2.
               To further define the structural requirements for the

25   interaction between peptide epitopes and various class I
alleles of interest, analogs of 10 residues in length of some
of the 9 residue peptides shown in Table 13 were synthesized
(Table 14). These analogs were generated by inserting an
additional Ala residue within the poly A backbone, so that the

30   anchor residues are not located in positions 2 and 10 (as
opposed to 2 and 9 in the previous Table). The results
obtained illustrate that motifs of 10 residues are also
capable of specifically binding to the relevant class I
alleles, albeit with a slightly lower efficiency.

35   In summary, these data confirm that both 9-mer and
10-mer peptid s which contain the appr priate m tifs can bind
HLA. On the basis f these data, 8-mer r 11-mer peptides

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should also be capable of binding, even if perhaps with lower
affinities.

    The data described above show that the presence f
certain residues in the anchor positions does allow (at least
5   in a "neutral" poly A backbone) for HLA binding. To
investigate to what degree other amino acids (for example,
chemically related amino acids) might be tolerated in these
crucial anchor positions, analogs of some of the poly A
peptides from Table 13 were synthesized, in which the residue
10   present in position 2 (or 3) or 9 was varied. The results of
this analysis are shown in Tables 15-19.

    In the case of A3.2 (Table 15), in position 2, L, M,
I, V, S, A, T, and F were found to be preferred (binding ≥ 0.1
relative to previously defined anchor residues), while C, G,
15   and D were permitted (binding ≥ 0.01 to 0.1 relative to
previously defined anchor residues). The substiution of E,
because of its similarity to D, in this position should also
be tolerated. In position 9, K, R, and Y were preferred.
Because of a similarity in nature, that H and F should also be
20   preferred. No other residue was tolerated in position 9 for
A3 binding.

    In the case of A11 (Table 16), the preferred residues
in position 2 were L, M, I, V, A, S, T, G, N (L and Q by
similarity). Tolerated were C, F, D (and E by similarity).
25   In position 9, K was preferred and R was tolerated. H should
also be tolerated by similarity.

    In the case of A24 (Table 17), Y and F were preferred
in position 2 (and W by similarity); no other residue was
tolerated. In position 9, F, I, and L were preferred (and W
30   and M by extension). No other residue was tolerated.

    In the case of A1, three different anchor residues
had previously been defined. The results shown in the
preceding section show that they act independently of each
other (i.e., that two out of three anchors would be sufficient
35   for binding). This is indeed the case. For this reason,
analogs containing tw anchors were synthesized t defin what
residues might be preferred or t l rated in each p sition.
The data sh wn in Table 18 show that in positi n 2, T, S, and

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M are preferred, and no other residue is t lerated. In
p siti n 3 (Table 19), D and E are preferred, and A,S (and T
by similarity) ar t lerated. Finally, in p siti n 9, nly Y
is preferred, and no other residue appears to be tolerated
5   (Table 19).

     Thus, on the basis of the data, it is concluded that
peptides carrying any combination of two preferred residues
can bind.   Peptides containing "imperfect" motifs, i.e.,
carrying a preferred residue at one position and a tolerated
10 one at the other anchor position, should also be capable of
binding, even if with somewhat lower affinity. Using the
motifs of this invention for various MHC class I alleles amino
acid sequences from various viral and tumor-related proteins
were analyzed for the presence of motifs. The results of this
15 motif analysis is shown in Table 23 a - k.

                         Example 11
    Validation of HLA Peptide Binding Motifs with an Unbiased Set
                      of HPV 16 Peptides.
20   Human Papillomaviruses (HPVs) are implicated in the
etiology of cervical cancer (Pfister, H. (1974) Biology and
biochemistry of papillomaviruses, Rev. Physiol. Biochem.
99:111; zur Hausen, H. (1991). Human papillomaviruses in the
pathogenesis of anogenital cancer. Virology. 184:9) and in up
25 to 10% of total mortality due to cancer worldwide (zur Hausen,
H. (1991). Viruses in Human Cancers. Science, 254:1167).
Cervical cancer is the second most common cause of
cancer-related death in females worldwide (Parkin, D. M.,
Laara, E., and Muir, C. S. (1988), Estimates of the worldwide
30 frequency of sixteen major cancers in (1980). Int. J. Cancer.
41:184). HPV DNA is present in more than 90% of the cervical
carcinomas and predominantly of the HPV 16 genotype (Resnick,
R. M., Cornelissen, M. T., Wright, D, K., Eichinger, G. H.,
Fox, H. S., ter Schegget, J., and Manos, M. M. (1990).
35 Detection and typing of human papillomavirus in archival
cervical cancer specimens by DNA amplification with c nsensus
primers. J. Natl. Cancer Inst; Van den Brule, A. J. C.,
Walboomers, J. M. M., du Maine, M., Kenemans, P., and Meijer,

C. J. L. M. (1991). Difference in prevalence of human
papillomavirus gen types in cytom rph logically normal sm ars
is ass ciated with a history f cervical intraepithetal
neoplasia. *Int. J. Cancer.* 48:404). The ability of HPV 16

5 early region 6 and 7 (E6, E7) open reading frames to in vitro
immortalize rodent cells (Yasumoto, S., Burkhardt, A.L.,
Doniger, J., and DiPaolo, J.A. (1986). Human Papillomaviruses
type 16 DNA induced malignant transformation of NIH3T3 cells.
J. Virol. 57:572) and human keratinocytes (Pirisi. L.,

10 Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J.A.
(1987). Trans-formation of human fibroblasts and
keratinocytes with human papillomavirus type 16 DNA. *J.
Virol,* 61:1061) and to transform human fibroblasts (Smits, H.
L., Raadsheer, E., Rood, I., Mehendale, S., Slater, R. M., van

15 der Noordaa, J., and ter Schegget, J. (1988). Induction of
anchorage-independent growth of human embryonic fibroblasts
with a deletion in the short arm of chromosome 11. *J. Virol.*
62:4538) suggests direct involvement of HPV 16 in the
multi-step process of cervical carcinogenesis,

20   In general T cell immunity, in particular mediated by
cytotoxic T lymphocytes (CTL) is important in the defense
against virus-induced tumors (Melief, C. J. (1992). Tumor
eradication by adoptive transfer of cytotoxic T lymphocytes.
Adv. Cancer Res. 58:143; Melief, C. J., and Kast, W. M.

25 (1992). Lessons from T cell responses to virus induced tumors
for cancer eradication in general. *Cancer Surv.* 13:81).
Recently in a mouse model, it was reported that some degree of
protection against HPV 16 E7 expressing tumors can be obtained
with CTL after immunization with HPV 16 E7 expressing cells

30 (Chen. L., Thomas, E, K., Hu, S. L., Hellström, I., and
Hellstrom, K. E. (1991). Human papillomavirus type 16
nucleoprotein E7 is a tumor rejection antigen. *Proc. Natl.
Acad. Sci.* 88:110; Chen, L., Ashe, S., Brady, W. A.,
Hellstrom, I., Hellström, K. E., Ledbetter, J. A,, McGowan,

35 P., and Linsley, P. S. (1992). Costimulation of Antitumor
immunity by the B7 c unterreceptor f r the T lymphocyt
molecules CD28 and CTLA-4. *C 11.* 71:1093). In viv
protecti n by CTL was recently sh wn in mouse m dels in which

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synthetic peptides containing CTL pitopes were used f r
efficient priming f mice against virus infe ti ns (Schulz,
M., Zinkernagel, R. M., and Hengarter, H. (1991).
Peptide-induced antiviral protection by cytotoxic T cells.
5   *Proc. Natl. Acad. Sci. USA* 88:991; Kast, W. M., Roux, L.,
Curren, J., Blom, H. J. J., Voordouw, A. C., Meleon, R. H.,
Kolakofski, D., and Melief, C. J. M. (1991). Protection
against lethal Sendai virus infection by in vivo priming of
virus-specific cytotoxic T lymphocytes with an unbound
10   peptide. *Proc. Natl. Acad. Sci. USA.* 88:2283). Moreover in a
mouse model it has now been shown that complete protection
against HPV 16 induced tumors can be achieved by peptide
vaccination with a CTL epitope derived from the viral oncogene
E7().

15   The HPV 16 E6 and E7 gene products are the most
desirable target antigens for vaccination against HPV 16
induced tumors. Both are retained and highly expressed in HPV
16-transformed cancer cells in vivo (Baker, C. J., Phelps, W.
C., Lindgren, V., Braun, M. J., Gonda, M. A., and Howley, P.
20   M. [1987]. Structural and transcriptional analysis of human
papillomavirus type 16 sequences in cervical carcinoma cell
lines. *J. Virol.* 61:962; Smotkin, D., and Wettstein, F. O.
[1986]. Transcription of human papillomavirus type 16 early
genes in a cervical cancer and cancer-derived cell line and
25   identification of the E7 protein. *Proc. Natl. Acad. Sci. USA.*
83:4680) and involved in the induction and maintenance of
cellular transformation in vitro (Crook, T., Morgenstern, J.
P., Crawford, L., and Banks, L. [1989]. Continued expression
of HPV-16 E7 protein is required for maintenance of the
30   transformed phenotype of cells co-transformed by HPV-16 plus
EJ *ras*. *EMBO J.* 8:513; Hawley-Nelson, P., Vousden, K. H.,
Hubbert, N. L., Lowy, D. R., and Schiller, J. T. [1989]. HPV
16 E6 and E7 proteins cooperate to immortalize human foreskin
keratinocytes. *EMBO J.* 8:3905). Dependence of in vitro growth
35   of cell lines derived from cervical cancers on the expression
 f E6 and E7 emphasizes inv lvement of these ncogenes in
maintenanc of the phen type of cervical carcin ma cell lines
(V n Knebel D beritz, M,, Bauknect, T., Bartch, D., and zur

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Hausen, H. [1991]. Influence of chromosomal integration n glucoc rticoid-regulat d transcripti n of growth-stimulation papillomavirus genes E6 and E7 in cervical carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 88:1411). To determine the CTL epitopes and potential vaccine candidates of HPV 16 for humans, we screened peptides spanning the HPV 16 E6 and E7 protein sequences for their ability to bind to the most frequent human MHC molecules, namely HLA-A1, A3.2, A11.2 and A24. Combined these five alleles will cover about 90% of the world population (Dupont, B., ed. [1987]. Immunology of HLA Vol. I -- Histocompatibility Testing. Springer-Verlag, New York).

A complete set of 240 overlapping synthetic peptides of 9 aa length and 8 aa overlap covering the entire HPV 16 E6 and E7 oncogene sequences were synthesized. The peptides were tested for their ability to bind the aforementioned HLA molecules in the binding assay described above. The results of this analysis show the relative affinity of all peptides for the respective HLA alleles and reveal the possible candidate CTL epitopes for use in peptide based vaccines for humans in Tables 20(a)-(d).

The results confirm that peptide binding motif described in this invention for the aforementioned HLA alleles predict which peptide of a protein is likely to bind into the groove of a specified HLA molecule. Since we used a large and unbiased set of peptides, the results of the peptide binding analyses were used to evaluate the value of these motifs both for their predictive capacities and the necessity to have particular anchor aa residues on positions 2, (3) and 9 in a peptide.

Peptides. Peptides were generated by solid phase strategies on a multiple peptide synthesizer (Abimed AMS 422) by repeated cycles in which addition of Fmoc protected amino acids to a resin of polystyrene was alternated with a Fmoc-deprotection procedure (Gausepohl, H., Kraft, M., Boulin, Ch., and Frank, R. W. [1990]. Automated multiple peptide synthesis with BOP activation. *in* Pr c. f the 11th American peptide symp sium. J. E. Rivier and G. R. Marshall, Ed.

ESCOM, Leiden. 1003-1004). Th peptides all carrying a COOH
gr up at the C-terminal end, were cleaved fr m the resin and
sid chain protectiv gr ups were removed by treatment with
aqueous TFA. Peptides were analyzed by reversed phase HPLC
5 lyophilized and dissolved at a concentration of 1 mg/ml in
phosphate-buffered saline with 3% DMSO (Sigma, St. Louis, MO
63175) before use. Once dissolved, the peptides were stored
at -70° C. Since cysteine containing peptides are susceptible
to (air) oxidation during synthesis and handling, these
10 peptides were synthesized with an alanine instead of a
cysteine.

        *Identification of peptides from HPV 16 E6 and E7
proteins that bind to different HLA-A alleles.* A complete set
of 240 peptides of 9 aa in length and overlapping by 8 aa,
15 covering the sequences of the entire HPV 16 E6 and E7
]proteins, was tested for binding to 5 different HLA-A
molecules.

        The results of this analysis are depicted in Tables
20(a)-(d). Table 20(a) describes the peptides of HPV 16 that
20 bound to HLA-AL molecules. All peptides were tested. Listed
are only peptides yielding ratio values of $\geq$ 0.001. It can be
seen that 2 peptides bound with high affinity to this molecule
(>0.1), 6 with intermediate affinity (0.1-0.01) and 1 with low
affinity (0.01-0.001). Peptides were ranked by ratio value to
25 allow comparison of data obtained in different experiments.
To calculate the concentration of a peptide necessary to yield
a 50% inhibition dose (IC_{50}) one has to divide the value of
the standard IC_{50} by the ratio. For example, peptide E6-80
has an IC_{50} of 23 nM (81/3.5).
30   Table 20(b) describes the peptides that bound to HLA-
A3.2 molecules. Seven peptides were identified as high
affinity binders, 6 as intermediate affinity binders and 13 as
low affinity binders. Table 20(c) describes the peptides that
bound to HLA-A11.2 molecules. Six high affinity peptides were
35 identified, 4 intermediate affinity binders and 10 low
affinity binders. Tw high affinity binding peptides (E6-59
IVYRDGNPY and E6-80 ISEYRHYAY) and tw weak affinity binding
peptid s with a Y at the 9th positi n (E6-42 QQLLRREVY, E6-69

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VADKALKFY) were identified for HLA-A11.2 Considering the high
binding strength of the first tw peptides and the similarity
between the HLA-A11.2 m tif and the HLA-A3.2 motif in which
Y's are preferred at the 9th aa position, tyrosines should be
included at the 9th position in the HLA-A11.2 motif. Comparing
Tables 21(b) and (c) it is clear that there is a large overlap
of peptides that bound to both A3.2 and A11.2 molecules.
Eighteen out of 28 E6 and E7 peptides binding to these two HLA
molecules overlapped and only 8 peptides were unique for HLA-
A3.2 and 2 peptides unique for HLA-A11.2.

Finally, Table 20(d) describes the peptides that
bound to HLA-A24 molecules. Here 2 peptides were identified
as high affinity binding peptides, 5 as intermediate affinity
binding peptides and 5 as low binding peptides. One high
affinity peptide (E6-72 KALKFYSKI) and one intermediate
affinity peptide (E7-49 RAHYNIVTF) were identified, indicating
that an A at the second position should be allowed in the HLA-
A24 motif. All these inclusions are indicated in Table 20-e.
In analyzing these tables it can be concluded that between 2
and 7 high affinity binding peptides were identified for all
of the tested HLA-A molecules. Occasionally some peptides
were binding to more alleles. Three peptides (E6-7, E6-37 and
E6-79), bound to HLA-A2.1, A3.2 and A11.2. One peptide (E6-
38) bound to HLA-A3.2, A11.2 and A24 and two peptides (E6-69
and E6-80) bound to HLA-A1, A3.2 and A11.2. But these
crossreactive peptides bound only weakly to one or more of the
different HLA molecules. In general, however, it can be
concluded that, except for HLA-A3.2 and HLA-A11.2 molecules,
almost all HLA molecules bind unique peptides.

*Validation of HLA-A peptide binding motifs with an
unbiased set of HPV 16 E6 and E7 peptides.*

We analyzed how well the motifs for anchor positions
described in this invention predicted the binding of a
peptide, and also the reverse: how well binding peptides
followed the identified motifs. For this, peptides were
ranked as high binders, intermediate binders, weak binders,
and negative binders and for each peptide the m tif prediction
based n the anchor motif rules of Table 6 were analyzed. The

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overall efficiency f the 2, (3), and 9 anch r motifs was then
calculated and this is summarized in Table 20(e). It can be
c ncluded that th m tif described above for the different
HLA-A molecules are quite accurate. One hundred percent of
5   the HLA-A1, A3.2, and A24 high binders would be predicted as
well as 67% of the HLA-11.2. Even for the intermediate
binders between 40 and 100% would be predicted depending on
the HLA-A molecule analyzed. Furthermore, the percent of weak
binding peptides that would be predicted is low and the
10   percent of those peptides that were predicted to bind but
actually did not bind is very low for all these alleles.

          Analyzed differently, of the 12 peptides predicted to
bind to HLA-A1 actually 5 bound with high or intermediate
affinity. This indicates that only a few peptides would have
15   to be made to find these potential CTL epitopes. The figures
for HLA-A3.2, A11.2, and A24 were 10/32, 7/26, and 4/7,
respectively. This implies that the predictive value for all
of these alleles is good. Besides a small number of peptides
that had not been predicted by the recently described motifs,
20   the (-) in Tables 21(a) - (d), a number of peptides that were
predicted by the 2, (3) and 9 anchor motifs did not bind,
indicating that having the right anchor residues is not always
sufficient for binding and implicating that non-anchor
residues can make negative contributions to the binding of a
25   peptide.

                            Example 12
                 Presence of a Motif is Necessary But Not
                 Sufficient for High Affinity Class I Binding
30   To investigate further how the presence of different
motifs might influence the capacity of different peptides to
bind to the relevant HLA alleles, the sequences of various
potential target molecules were scanned for the presence of
motif-containing peptides. The peptides thus identified were
35   synthesized and tested for binding. It was f und (Table 20)
that in the cas   f A3.2, only 39 (19%) f the 205 peptides
b und with high affinity in the 1 to 50 nM range. 22.4% of
them b und with intermediate affinities (in the 50 t 500 nM

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range), while 34.6% bound weakly (in the 500 nM to 50 μM
range). Finally, 23.9% f them did not bind at all, at least
up t the 50 μM level. In the case of A11, 33 (33%) of the
100 peptides bound with high affinity in the 1 to 50 nM range.
5   35% of them bound with intermediate affinities (in the 50 nM
range.), while 24% bound weakly (in the 500 nM to 50 μM
range). Finally, 8% of them did not bind at all, at least up
to the 50 μM level.

              Similar results were also obtained (data not shown)
10   in the case of A1 and A24.

              The same type of analysis were also performed in the
case of 10-mer peptides carrying either the A3.2, and A11
motifs (Tables 22(a) and (b)). It was found that in these
cases, the frequency of good binders was even lower (17.5%,
15   and 29.8%, respectively). These data confirm the fact that
motif-containing 10-mer peptides can indeed bind, albeit with,
in general, reduced affinity.

              In summary, the data shown in this section clearly
show that the presence of the correct anchor residues is not
20   sufficient per se to allow for good HLA binding. It is thus
apparent that the nature of the residues contained in
positions other than 2(3) and 9 (or 10) can influence binding.
The most likely explanation of this observation is that the
presence of certain residues (in positions other than 2 and 9)
25   can negate or increase the binding potential of a peptide
determinant.

              The data shown in the preceding sections describe how
specific binding assays can be used to identify, within motif-
containing peptides, peptides that are immunogenic. We also
30   wanted to devise an alternative strategy, namely to derive
procedures that would be able to predict, within motif-
containing peptides, which peptides might be good or
intermediate binders and thereby might be immunogenic. In
other experiments not shown intermediate or good binders have
35   been shown to be immunogenic. In particular, t identify
r sidues that have a negative impact on binding an analysis f
all p sitions for A3.2, A11, and all m tif-containing
p ptides, b th 9-mers and 10-mers is carried out. In th case

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f All, because of the small ccurrence f n nbinding
peptides, a different cut ff was used such that th analysis
c mpares go d and intermediate binders on the one hand to weak
and nonbinders on the other.

5

Example 13
Algorithms to Identify Immunogenic Peptides

In light of results presented in Example 13 above,
algorithms are developed to provide a more exact predictor of
binding based upon the effects of different residues at each
position of a peptide sequence, in addition to the anchor or
conserved residues. More specifically, we utilize the data
bank obtained during the screening of our collection of A1,
3,11 or 24 motif containing peptides to develop an algorithm
for each particular allele which assigns a score for each
amino acid at each position along a peptide. The score for
each residue is taken as the ratio of the frequency of that
residue in good and intermediate binders to the frequency of
occurrence of that residue in non-binders.

In the present algorithm residues have been grouped
by similarity. This avoids the problem encountered with some
rare residues, such as tryptophan, where there are too few
occurrences to obtain a statistically significant ratio. A
listing is made of scores obtained by grouping for each of the
twenty amino acids by position for 9-mer peptides containing
conserved residues that define their motif (2/9 motifs). A
peptide is scored in the algorithm as a product of the scores
of each of its residues.

The power of an algorithm to correlate with binding
is further underlined by its ability to predict a population
of peptides with the highest occurrence of good binders. If
one were to rely, for example, solely on the 2/9 motif for
predicting 9-mer peptides which bind to a specific MHC allele
the large number of peptides containing the motif would be
predicted to be good binders. In fact only a relativ ly small
percentage of th se peptides are good binders and a s mewhat
larger percentage are intermediate bind rs, while a still
larger percentage of the peptides predicted by the motif are

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either weak or non-binding peptides. In contrast, using the gr uped alg rithm of this inv nti n a population of peptides are created with a greater percentage f g d binders, a still greater percentage of intermediate binders, and a smaller percentage, relative to that predicted by motif-containing peptides, are weak and non-binders.

The present example of an algorithm uses the ratio of the frequency of occurrence of an amino acid in binders and non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill in the art that there are alternative ways of creating a similar algorithm. For example, one could use average binding affinity values, or relative binding of single amino acid substitutions in a motif containing peptide with a poly-alanine backbone to generate an algorithm table.

An algorithm using average binding affinity has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler group ratio algorithm. We create such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of motif containing peptides. The algorithm score for a peptide is then taken as the sum of the scores by position for each of its residues.

Example 14
Preparation of effective HLA allele-specific antigen presenting cells.

This example demonstrates the use of cold temperature incubation or acid stripping/peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC). The APC were used to sensitize precursor cytotoxic T lymphocytes which led to the development of antigen-specific cytotoxic cells. This was accomplished using either phytohemaglutinin (PHA) T-cell blasts or peripheral blood m n nuclear cells (PBMC) r staphyloc ccus aureus C wan I (SAC-I) activated PBMC as APC. The results are applicable to other APC and to the other MHC alleles.

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The f ll wing describes s urces for materials used in the f llowing examples:

L-Asc rbic acid, Cat #B582, J.T. Baker, Phillipsburg, NJ.

Anti-HLA A2 (BB7.2), Cat #HB82, ATCC, Rockville, MD.

Anti-HLA DR (LB3.1), from J. Gorga, Children's Hospital, Pittsburgh, PA.

Anti-HLA Alpha chain pan ABC (9.12.1), from R. DeMars, University of Wisconsin, Madison, Wl.

Anti-mouse IgG FITC conjugate, Cat #F2883, Sigma, St. Louis, MO.

$\beta_2$microglobulin, Cat #MO114, Scripps Labs, San Diego, CA.

BSA Fraction V, Cat #A9418, Sigma, St. Louis, MO.

50cc conical centrifuge tubes, Cat #2070, Falcon, Lincoln, Park, NJ.

Cryo 1°C freezing container, Cat #5100-0001, Nalge, Rochester, NY.

Cryovial, Cat #5000-0012, Nalge, Rochester, NY.

Dimethyl sulfoxide (DMSO), Cat #D2650, Sigma, St. Louis, MO.

DNAse, Cat #260912, Calbiochem, San Diego, CA.

Dynabeads M-450 goat anti-mouse IgG, Cat #110.06, Dynal, Great Neck, NY.

EDTA tetrasodium salt, Cat #ED4SS, Sigma, St. Louis, MO.

FACScan, Becton Dickinson, San Jose, CA.

Fetal calf serum (FCS), Cat #3000, Irvine Scientific, Irvine, CA.

Ficoll-Paque, Cat #17-0840-03, Pharmacia, Piscataway, NJ.

Gentamicin, Cat #600-5750AD, Gibco, Grand Island, NY.

L-Glutamine, Cat #9317, Irvine Scientific, Irvine, CA.

GS-6KR centrifuge, Beckman Instruments, Palo Alto, CA.

Human AB serum (HS), Cat #100-112, Gemini Bi products, Calabasas, CA.

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Human rIL-2, Sand z, Basel, Switzerland.

Human rIL-7, Cat #F1-1587-1, Genzyme, Cambridge, MA.

Is pr panol, Cat #A464-4, Fisher Scientific, Pittsburgh, PA.

5   MicroCELLector T-150 culture flask for selection of CD4+ cells, Cat #8030, Applied Immune Sciences, Menlo Park, CA.

Micromedic automatic gamma counter, ICN Micromedics Systems, Huntsville, AL.

10   OKT4 hybridoma supernatant, Cat #CRL 8002, ATCC, Rockville, MD.

Paraformaldehyde, Cat #T-353, Fisher, Pittsburgh, PA.

PBS calcium and magnesium free (CMF), Cat #17-516B, BioWhittaker, Walkersville, MD.

15   Peptides used in this study were synthesized at Cytel and described in Table 24 a.

Phytohemagglutinin (PHA), Cat #HA-16, Wellcome, Dartford, England.

RPMI 1640 + Hepes + glutamine, Cat #12-115B, 20   BioWhittaker, Walkersville, MD.

RPMI 1640 + Hepes + glutamine, Cat #380-2400AJ, Gibco, Grand Island, NY.

Sodium chloride (NaCl), Cat #3624-05, J.T. Baker, Phillipsburg, NJ.

25   Sodium (^{51}Cr) chromate, Cat #NEZ 030, NEN, Wilmington, DE.

Sodium phosphate monobasic, Cat #S9638, Sigma, St. Louis, MO.

Triton X-100, Cat #X-100, Sigma, St. Louis, MO.

30   24 well tissue culture plate, Cat #3047, Falcon, Becton Dickinson, San Jose, CA.

96 well U-bottomed cluster plate, Cat #3799, Costar, Cambridge, MA.

35   *Culture Medium.* PHA blasts and CTL inductions were done in RPMI 1640 + Hepes + glutamine (Gibc) supplem nted with 2 mM L-glutamine (Irvine Scientific), 50 μg/ml gentamicin (Gibco), and 5% heat inactivated pooled human Type AB serum (Gemini

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Bi products) [RPMI/5% HS]. EBV transformed lymph blast id
cell lines (LCL) were maintain d in RPMI 1640 + Hepes +
glutamine (BioWhittaker) supplemented with L-glutamine and
gentamicin as above and 10% heat inactivated fetal calf serum
5   (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays
were performed in RPMI/10% FCS.

Cytokines. Recombinant human interleukin-2 (rIL-2) (Sandoz)
was used at a final concentration of 10 U/ml. Recombinant
10   human interleukin-7 (rIL-7) (Genzyme) was used at a final
concentration of 10 ng/ml.

Isolation of Peripheral Blood Mononuclear Cells (PBMC). Whole
blood was collected in heparin (10 U/ml) containing syringes
15   and spun in 50cc conical centrifuge tubes (Falcon) at 1600 rpm
(Beckman GS-6KR) 15 min. The plasma layer was then removed
and 10 ml of the buffy coat collected with a 10 ml pipette
using a circular motion. The buffy coat was mixed thoroughly
and diluted with an equal volume of serum free RPMI 1640. The
20   diluted buffy coat was then layered over 20 ml Ficoll-Paque
(Pharmacia) in a 50cc conical tube and centrifuged 400 x g for
20 min at room temperature with the brake off. The
Ficoll-plasma interface containing the PBMCs was collected
using a transfer pipet (two interfaces per 50cc tube) and
25   washed three times with 50 ml RPMI (1700, 1500, and 1300 rpm
for 10 min.

Freezing and Thawing PBMC. PBMC were frozen at 30×10^6
cells/ml of 90% FCS + 10% DMSO (Sigma), in 1 ml aliquots using
30   cyrovials (Nalge). Cryovials were placed in Cryo 1°C freezing
containers (Nalge) containing isopropanol (Fisher) and placed
at -70°C from 4 hr (minimum) to overnight (maximum).
Isopropanol was changed after every 5 uses. Cryovials were
transferred to liquid nitrogen for long term storage. PBMC
35   were thawed by continu us shaking in a 37°C water bath until
the last crystal was nearly thawed. Cells were immediately
diluted int serum free RPMI medium c ntaining DNAs 30 μg/ml
(to av id clumping) (Calbiochem), and washed twice.

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Depl tion of Lymphocyte Subpopulations. CD4 lymphocyte
depleti n was perf rmed using antibody-c ated flasks:
Micr CELLect r T-150 flasks f r the selection f CD4+ cells
(Applied Immune Sciences) were washed according to the
5   manufacturer's instructions with 25 ml PBS CMF + 1 mM EDTA
(Sigma) by swirling flasks for 30 sec followed by incubation
for 1 hr at room temperature on a flat surface. Buffer was
aspirated and flasks were washed 2 additional times by shaking
the flasks for 30 sec and maintaining coverage of the binding
10 surface. To each washed flask, 25 ml culture medium + 5% HS
were added and incubated for 20 min at room temperature on a
flat surface. Media was left in the flask until it was ready
to receive the cells. PBMC were thawed in RPMI/5% HS
containing 30 μg/ml DNAse, and washed twice. HS in the wash
15 blocks Fc receptors on PBMCS. For one flask a maximum of 12 x
10^7 cells were resuspended in 25 ml culture medium. Culture
medium was aspirated from the flask and then the cell
suspension was gently added to the MicroCELLector. Flasks
containing the cells were incubated for 1 hr at room
20 temperature on a flat surface. At the end of the incubation,
the flask was gently rocked from side to side for 10 sec to
resuspend the nonadherent cells. Nonadherent CD4 depleted
cells were harvested, and then flasks were washed twice with
PBS CMF to collect the nonadherent cells. Harvested
25 CD4-depleted cells were pelleted by centrifugation and
resuspended in complete culture medium (RPMI/5%/HS).

Generation of PHA Blasts. PBMC were isolated using the
standard Ficoll-Paque protocol. Frozen cells were washed
30 twice before use. Cells were cultured at 2 x 10^6/ml in
RPMI/5% HS containing 1 μg/ml PHA (Wellcome) and 10 U/ml
rIL-2. PHA blasts were maintained in culture medium
containing 10 U/ml r IL-2 with feeding and splitting as
needed. PHA blasts were used as APC on day 6 of culture.
35 Generation of empty class I molecules and peptide loading w re
 nly performed by the acid strip m thod when using these APC.
Acid Stripping/Peptid Loading of PBMC and PHA Blasts. PBMC
were isolated using the Ficoll-Paque protocol. When using

fr zen cells, PBMC were washed twice bef re using. PHA blasts
were prepared as previ usly described and washed twic bef re
using. Once cells were prepared, they were washed once in
cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc

5   conical centrifuge tube, the cells were resuspended at 10^7/ml
in cold sterile citrate-phosphate buffer [0-13 M L-ascorbic
acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma)
pH 3, 1% BSA, 3 μg/ml $\beta_2$microglobulin (Scripps Labs)] and
incubated for 2 min on ice. Immediately, 5 volumes of cold

10   sterile neutralizing buffer #1 [0.15 M sodium phosphate
monobasic pH 7.5, 1% BSA, 3 μg/ml $\beta_2$microglobulin, 10 μg/ml
peptide] were added, and the cells were pelleted at 1500 rpm,
5 min at 4°C. Cells were resuspended in 1 volume cold sterile
neutralizing buffer #2 [PBS CMF, 1% BSA, 30 lig/ml DNAse, 3

15   μg/ml $\beta_2$microglobulin, 40 μg/ml peptide] and incubated for 4
hrs at 20°C. Cells were diluted with culture medium to
approximately 5 x 10^6/ml and irradiated with 6000 rads. Cells
were then centrifuged at 1500 rpm for 5 min at room
temperature and resuspended in culture medium. The acid

20   stripped/peptide loaded cells were used immediately in the CTL
induction cultures (below).

*Induction of Primary CTL using Acid Stripped/Peptide Loaded
Autologous PBMCs or PHA Blasts as Stimulators.* Acid

25   stripping/peptide loading of PBMC and PHA blasts are described
above. During the last 4 hr incubation of stimulator cells
with peptide, the responder cell population was prepared:
Responders were PBMC that were depleted of CD4+ cells
(described above). Responder cells were resuspended in

30   culture medium at 3 x 10^6/ml. 1 ml of the responder cell
suspension was dispensed into each well of a 24-well tissue
culture plate (Falcon, Becton Dickinson). The plates were
placed in the incubator at 37°C, 5% CO_2 until the stimulator
population was ready. Once irradiated, stimulator APC were

35   resuspended in culture medium containing 20 ng/ml rIL-7 at
10^6/ml for the PBMC, or at 3 x 10^5/ml f r the PHA blasts. 1
ml of stimulat r cell suspension was added per well t the
plates c ntaining the responders. On day 7 after induction, a

100 μl culture medium containing 200 ng/ml rIL-7 was added to each well (20 ng/well rIL-7 final). On day 10 after inducti n, 100 μl f culture medium containing 200 U/ml rIL-2 was added to each well (20 U/well rIL-2 final).

Antigen Restimulation of CTL. On day 12-14 after the induction, the primary CTL were restimulated with peptide using adherent APC. Autologous PBMC were thawed and washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium at 4×10^6/ml. 1 ml of cell suspension was added to each well of a 24-well tissue culture plate, and incubated for 2 hrs at 37°C, 5% CO_2. Non-adherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μg/ml $\beta_2$microglobulin and 20 μg/ml total peptide was added to each well. APC were incubated for 2 hrs at 37°C, under 5% CO_2 with the peptide and $\beta_2$microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5×10^6/ml in culture medium was added to each well. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well.

Cytotoxicity Chromium Release Assay. Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.

       a.   <u>Effector Cell Preparation</u>: the responders, which at this stage are renamed "effectors", were centrifuged and resuspended at 10^7/ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μl/well on 96 well U-bottomed cluster plates (Costar), in duplicate.

       b.   <u>Target Cell Preparation</u>: Approximately 16-20 hrs prior to the assay, target cells were resuspended at 3×10^5/ml in RPMI/10% FCS in the presence or absence of 3 μg/ml $\beta_2$micr globulin and 10 μg/ml t tal peptid . After preincubati n, target cells were centrifuged and pellets were resuspended in 200 μl (300μCi) sodium (^{51}Cr) chromate (NEN).

Cells were incubated at 37°C for 1 hr with agitation.
Labelled target cells were wash d 3 times with RPMI/10% FCS.

       c. Setting-Up the Assays: Target cell c nc ntrati n
was adjusted to 10^5/ml in RPMI/10% FCS and 100 μl aliquots

5   were added to each well containing responders. K562 cells
(cold targets, to block NK, and LAK activity) were washed and
resuspended in RPMI/10% FCS at 10^7/ml. Aliquots of 20 μl were
added per well, yielding a 20:1 of cold K562 target:labelled
target. For the determination of the spontaneous ^{51}Cr

10   release, 100 μl/well of RPMI/10% FCS were added to 100 μl/well
of labelled target cells, and 20 μl/well of K562. For maximum
^{51}Cr release, 100 μl 1% Triton X-100 (Sigma) in PBS CMF, was
added to the 100 μl/well labelled target cells, and 20 μl/well
K562. Plates were centrifuged for 2 min at 1200 rpm to

15   accelerate cell conjugate formation. Assays were incubated
for 5 hr at 37°C, 5% CO_2. Assays were harvested by
centrifuging plates for 5 min at 1200 rpm and collecting 100
μl/well of supernatant. Standard gamma counting techniques
were used to determine percent specific lysis (Micromedic

20   automatic gamma counter, 0.5 min per tube).

Cultured Cell Lines. JY, a HLA A2.1 expressing human
EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562,
a NK cell sensitive erythroblastoma line was grown in RPMI/10%

25   FCS. K562 was used to reduce background killing by NK and LAK
cells in the chromium release assays.

Peptides. The peptides used in these studies were synthesized
at Cytel and their sequences are described in Table 24 a.

30   Peptides were routinely diluted in 100% DMSO at 20 mg/ml,
aliquoted, and stored at -20°C.

FACS Analysis. Approximately 10^6 cells were used for each
antibody that was to be tested. Cells were washed twice with

35   PBS CNU + 0.1% BSA. To each sample, 100 μl PBS CMF + 0.1% BSA
+ primary antibody at 2 μg/ml (BB7.2, ATCC) or (9.12.1,
Inserm-CNRS, Marseille, France) r (LB3.1, Children's Hospital
Pittsburgh) were added. A negative control was always

included. Cells were incubated on ice for 20 min and washed
twice with PBS CMF + 0.1% BSA. Cells were resuspended in 100
µl anti-m use IgG FITC c njugat (Sigma), diluted 1:50 in PBS
CMF + 0.1% BSA, and incubated 20 min on ice. Cells were
5   washed twice with PBS CMF + 0.1% BSA, and resuspended in PBS
for FACScan (Becton Dickinson) analysis. When it was
necessary to postpone analysis to the subsequent days, the
cells were fixed with PBS/1% paraformaldehyde (Fisher) and
analyzed within one week.
10

Binding Assays Using Intact Cells and Radiolabelled Peptide.
JY cells were treated with citrate-phosphate buffer and
neutralizing buffer #1 as described above. JY control cells
were left untreated in tissue culture media. After treatment
15   both cell populations were washed twice with serum free RPMI
and loaded with ^{125}I-radiolabelled 941.01 (HBc15-27) peptide
(standard chloramine T iodination). To determine binding
specificity, 2×10^6 cells were resuspended in 200 µl
neutralizing buffer #2 (described above) containing
20   ^{125}I-941.01 (10^5 cpms) +/- 100 µg unlabelled 941.01. Cells
were incubated for 4 hrs at 20°C and washed twice with serum
free RPMI to remove free peptide. Cells were resuspended in
200 µl of serum free RPMI. In a microfuge tube the cell
suspension was layered over an 800 µl FCS and pelleted by
25   centrifugation for 5 sec. Supernatants were aspirated and the
radioactivity remaining in the pellet was measured (Micromedic
automatic gamma counter, 1 min per tube).

Example 15

30   ## Class I MHC molecule peptide stripping/loading by mild acid
treatment.

Mild acid solutions of pH 3 such as glycine or
citrate-phosphate buffers have been used by various groups to
identify endogenous peptides and to identify tumor associated
35   T cell epitopes. The treatment is unique in that only the MHC
class I molecul s are destabilized (and peptides released),
while all other surface antig ns remain intact including MHC
class II m lecules. Most imp rtantly, treatment f cells with

the mild acid s lutions f this example do n t affect the
cell's viability or m tab lic state. The mild acid treatment
is rapid since the stripping f endogenous peptides ccurs in
two minutes at 4°C and the APC is ready to perform its
5   function after the appropriate peptides are loaded. In this
example we utilized the technique to make peptide specific
APCs for the generation of primary antigen-specific CTL. The
resulting APC were efficient in inducing peptide-specific CD8+
CTL.

10

Measurements by FACS Analysis. PHA-induced T-cell blasts were
acid stripped/peptide loaded according to the methods
described in Example 15. The resulting cells were stained for
FACS analysis using anti-HLA-A2 (BB7.2) and anti-HLA alpha
15   chain-specific (9.12.1) monoclonal antibodies. Controls for
this experiment included the same cell population which was
not treated at pH 3 (but treated with PBS buffer at pH 7.2),
and with cells treated with citrate-phosphate buffer (to strip
the MHC) but neutralized in the absence of $\beta_2$microglobulin and
20   peptide. The results presented in Figure 15, indicate that
treatment of these cells with the citrate-phosphate (pH3)
buffer significantly reduced (10-fold) the reactivity of the
cells toward both anti-HLA class I antibodies alone
(anti-HLA-A2 and the alpha chain specific), but not towards a
25   monoclonal antibody specific for class II MHC molecules
(anti-HLA-DR). Most importantly, neutralization of the
acid-stripped cells in the presence of $\beta_2$microglobulin and
peptide resulted in preservation of a significant amount of
class I MHC antibody-reactive sites, with only a 2.5-fold
30   decrease in fluorescence intensity. Importantly, the
acid-treated cells remained viable, as measured by trypan blue
exclusion and forward/lateral FACS scatter analysis. Similar
results were obtained using EBV-transformed B cell lines,
fresh (or frozen) PBMC and other peptides (which bind to
35   either HLA-A2.1 or HLA-A1) (data not shown).

Binding of Radiolab l d Peptid s to Empty MHC Mol cules. T
determin the efficiency f peptide l ading using the c ld

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temperature incubati n or acid stripping/peptide l ading
pr t c l, JY cells (an HLA-A2.1 EBV-transf rmed B cell line)
were preincubated at 26°C overnight or acid-stripped t remove
the endogenous MHC-associated peptides and the loading of
5   exogenous peptide was determined using a ^{125}I-radiolabelled
HLA-A2.1 binding peptide. The specificity of this reaction
was determined by measuring the inhibition of labelled peptide
binding using a cold peptide of the same sequence. Results
presented in Table 24 b demonstrate that acid-treatment of the
10   cells increased significantly (approximately 10-fold) the
amount of labelled peptide binding to the JY cells.
Furthermore, the binding of labelled peptide was completely
blocked by the addition of the cold peptide, demonstrating
specific binding (data not shown).

15

*In Vitro Induction of Primary Antigen-Specific CTL Using Acid
Stripped/Peptide Loaded APCS*. Additional critical parameters
for the induction of primary CTL using both the cold
temperature incubation and acid strip protocol are: 1)
20   enrichment of CD8+ T-cells in the responder cell population
(or depletion of CD4+ T-cells), 2) addition of rIL-7 to the
CTL induction cultures from day 0, and 3) restimulation of the
cultures with antigen on day 12-14 using autologous adherent
cells pulsed with peptide.   Results presented in Figs. 16 and
25   17 show experiments performed using PBMC and PHA-induced T-
cell blasts as APC. Figure 18 shows experiments using PHA-
induced T-cell blasts as APC while Figure 19 shows the use of
PBMC as APC.

30   Example 16
          Screening peptides to identify CTL epitopes.
          In order to identify CTL epitopes, CTL was stimulated
by SAC-I activated PBMCs as APC. Cold temperature expression
of the MHC in which class 1 β-2-microglobulin complex is
35   unstable was utilized in addition to acid stripping to
gen rate PBMC APC.

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Compl t Cultur Medium. The tissue culture medium used in
this study c nsisted f RPMI 1640 with Hepes and L-glutamine
(Gibco) supplemented with 2 mM L-glutamine (Irvine
Scientific), 0.5mM sodium pyruvate (Gibco), 100 U/100 ug/ml
5   penicillin/streptomycin (Irvine), and 5% heat-inactivated
Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture
media used in the growth of EBV-transformed lines contained
10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine)
instead of human serum.

10

Cytokines. Recombinant human Interleukin-2 (rIL-2) and
Interleukin-4 (rIL-4) were obtained from Sandoz and used at a
final concentration of 10 U/ml and 10 ng/ml, respectively.
Human interferon-γ (IFN-γ) and recombinant human Interleukin-7
15   (rIL-7) were obtained from Genzyme and used at 20 U/ml and 10
ng/ml, respectively.

Peptides. Peptides were synthesized at Cytel and are
described in Table 24 a. Peptides were routinely diluted in
20   100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until
use.

Cell Lines. JY, Steinlin, EHM, BVR, and KT3 are homozygous
human EBV-transformed B cell lines expressing HLA $A_{2.1}$, A_1, A_3,
25   A_{11}, and A_{24}, respectively. They are grown in RPMI/10% FCS.
K562, an NK cell sensitive, erythoblastoma line grown in
RPMI/10% FCS, was used for reduction of background killing in
CTL assays. Melanoma cell lines either expressing the MAGE
antigen, mel 397 and mel 938, or not expressing the MAGE
30   antigen; mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs).
Whole blood was collected into heparin containing syringes and
spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15
35   minutes. The plasma layer was then rem ved and 10 ml f buffy
coat was c llected with a pipett using a circular m ti n.
Th buffy c at was mix d well and diluted with an equal volume
 f RPMI. The buffy coat (30 ml) was then layered on 20 ml f

Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg)
for 20 minutes, 25°C, with the brake ff. The interfac
b tween the Fic ll and the plasma c ntaining the PBMCs was
recovered with a transfer pipet (two interfaces per 50 ml
5   tube) and washed three times with 50 ml of RPMI (1700, 1500,
and 1300 RPM for 10 minutes). Cells were resuspended in 10-20
ml of culture medium, counted, and adjusted to the appropriate
concentration.

10   *Freezing PBMCs.* 30 million cells/tube (90% FCS/10% DMSO;
Sigma) were inserted into a Nalgene Cryo 1°C Freezing
Container containing isopropanol (Fisher) and placed at -70°C
from 4 hrs (minimum) to overnight (maximum). The isopropanol
was changed every five times. Tubes were transferred to
15   liquid nitrogen for long term storage. To thaw, PBMCs were
continuously shaken in a 37°C water bath until the last
crystal was almost thawed (tubes were not allowed to sit in
the water bath or at room temperature for any period of time).
Cells were diluted into serum-free RPMI containing 30 μg/ml
20   DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs
        a. Preparation of APCs: PBMCs were purified using the
standard Ficoll-Paque protocol and resuspended at 1 x 10^6/ml
25   in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells
expressing Protein A; Calbiochem), 20 μg/ml Immunobeads
(Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4.
Two ml of cells per well were plated in a 24-well plate
(Falcon, Becton Dickinson) and cultured at 37°C. After 3
30   days, the medium was removed and the cells were washed three
times followed by addition of RPMI/10% HS. The cells were
used after culturing for an additional 2 days in RPMI/10% HS.

        b. Expression of empty Class I molecules on the surface of
35   APCs and peptide loading of APCs.
            1. C ld temperature incubati n:
                a. Expression f empty MHC in APCs: The APCs

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wer adjusted t a c ncentration f 2×10^6/ml in complete
culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN-γ,
and 3 μg/ml β2-microglobulin (β_2m; Scripps Lab). The cells
were then incubated overnight at 26°C in the presence of 5%

5   CO_2. It should be noted that these cells only express a
fraction of Class I molecules in the empty state (~10%).

              b.   Peptide loading of APC stimulator cells:
Empty Class I expressing APCs were washed 1-2 times with
serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1

10 X 10^7 in serum-free RPMI containing 50 μg/ml total of the
peptide pool (i.e., 16.7 μg/ml of each peptide in a pool of
three; 25 μg/ml of each peptide in a pool of two; 50 μg/ml of
individual peptide), 30 μg/ml DNAse, and 3 μg/ml β_2m.
Following a 4 hour incubation at 20°C, the cells were

15 irradiated at 6100 rads (5×10^6/ ml; 25 million cells/tube),
washed and adjusted to the appropriate concentration for
addition to the induction culture (see below).

              2.   Acid stripping: This was used as an
alternative method for generating empty MHC on the surface of

20 the APCs. The SAC-I activated PBMCs were washed once in cold
0.9% sodium chloride (J.T. Baker) containing 1% BSA. The
cells were resuspended at 10^7/ml in cold citrate-phosphate
buffer (0.13M L-ascorbic acid [J.T. Baker], 0.06M sodium
phosphate monobasic [Sigma], pH3) containing 1% BSA and 3

25 μg/ml β_2m and incubated on ice. After 2 minutes, 5 volumes of
cold 0.15M sodium phosphate monobasic buffer, pH7.5,
containing 1% BSA, 3 μg/ml β_2m, and 10 μg/ml peptide
[neutralizing buffer #1] was added and the cells centrifuged
at 1500 RPM for 5 minutes at 4°C. The cells were resuspended

30 in 1 ml of cold PBS containing 1% BSA, 30 μg/ml DNase, 3 μg/ml
$\beta_2$microglobulin, and 50 μg/ml peptide [neutralizing buffer #2]
and incubated for 4 hours at 20°C. As above, subsequent to
the four hour incubation at 20°C, the cells were irradiated at
6100 rads (5×10^6/ ml; 25 million cells/tube), washed, then

35 adjusted to the appropriate concentration for addition to the
inducti n cultur (s e below).

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c.   <u>Preparation of the CD4+ depleted PBMC responder cell</u>
<u>population (depletion of lymphocyte sub-populations using</u>
<u>AIS flasks).</u> AIS Micr Cellector T-150 flasks (specific
for the depletion of CD4+ T cells; Menlo Park, CA) were
5   primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30
seconds so that all surfaces were moistened, and then
incubating with the binding surface down at room temperature
for 1 hour. Following this incubation, flasks were shaken
vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2
10   additional times with PBS and then incubated with 25 ml of
culture medium for 15 minutes. PBMCs were thawed in
serum-free RPMI (+ L-glutamine + Hepes) containing 30 μg/ml
DNAse, washed once, and incubated for 15 minutes in culture
medium. Following aspiration of culture medium from the
15   flasks, up to 180 million PBMCs were added in 25 ml of culture
medium containing 30 μg/ml DNAse. After 1 hour at room
temperature, the flasks were rocked gently for 10 seconds to
resuspend the nonadherent cells. The nonadherent cell
suspension containing the CD8+ T cells was collected and the
20   flasks were washed 2 times with PBS. The CD4+ T cell depleted
PBMCs were centrifuged and counted for addition to the
induction culture. The CD4+ and CD8+ phenotype of the CD4+
depleted cell population was determined by FACS analysis (see
below). In general, this technique resulted in a two-fold
25   enrichment for CD8+ T cells with an average of approximately
40-50% CD8+ T cells and 15-20% remaining CD4+ T cells
following depletion of CD4+ T cells. Depletion of CD4+ T
cells can also be accomplished by antibody and complement or
antibody coated magnetic beads (Dynabeads). Depletion of CD4+
30   T cells served the purpose of enriching CTLp and removing
cells which would complete for cell nutrients and may
interfere with CTLp expansion.

     d.   <u>Induction of primary CTL</u>. During the 4 hour peptide
loading of the stimulator APCs, CD4+ depleted PBMC to be used
35   as the responder population were prepar d utilizing AIS flasks
f r selection of CD8+ T cells through the depletion f CD4+ T
cells (above). The responder cells were plated at 3 x 10^6/ml
in a 1 ml v lume (24 well plat) and placed at 37°C until the

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peptide loaded stimulator APCs were prepared. The irradiated,
peptide loaded APCs were washed 1 time in serum-free RPMI (+
L-glutamine and Hepes), adjusted to 1×10^6/ml in complete
medium, and plated into a 24 well plate at 1 ml/plate: For
5   PBMC, 1×10^6 stimulator cells (1 ml volume) were plated into
the wells containing the responder cells; For SAC-I activated
PBMC and PHA blasts, 1 ml of 3×10^5/ml stimulator cells were
plated in each well. A final concentration of 10 µg/ml of
additional peptide was added in addition to 10 ng/ml final
10   concentration of rIL-7 (2 ml total volume). On day 7 an
additional 10 µg/ml rIL-7 was added to the culture and 10 U/ml
rIL-2 was added every 3 days thereafter. On day 12, the
cultures were restimulated with peptide pulsed adherent cells
and tested for cytolytic activity 7 days later (below).
15

Protocol for Restimulation of Primary CTL Using Adherent APC.
PBMCs were thawed into serum-free RPMI (+ L-glutamine and
Hepes) containing 30µg/ml DNAse, washed 2 times, and adjusted
to 5×10^6 /ml in culture medium containing DNAse. PBMCs (25
20   million cells/tube in 5 ml) were irradiated at 6100R. After 1
wash, the PBMCs were resuspended in culture medium and
adjusted to 4×10^6/ml. 1 ml of irradiated PBMCs was added
per well of a 24-well plate. The PBMC were incubated for 2
hours at 37°c, washed 3 times to remove non-adherent cells,
25   and cultured in medium containing 20 µg/ml total peptide and 3
µg/ml $\beta_2$microglobulin added in a 0.5 ml volume and again
incubated for 2 hours at 37°C. The peptide was aspirated and
1.5×10^6 responder cells resuspended in culture medium were
added in a 1 ml volume. After 2 days, 1 ml of culture medium
30   containing 20 U/ml rIL-2 was added.

FACS Analysis. One million cells/tube were centrifuged,
resuspended in 100 ul/tube PBS/0.1% BSA/0.02% sodium azide
(Sigma) plus 10 ul/tube directly conjugated antibody (Becton
35   Dickinson), and incubated on ice 15-20 minutes. Cells were
then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and
resuspended in PBS to analyze on FACScan (Beckton Dickinson).
When it was not possible to analyze samples within 1-2 days,

c lls were fixed with PBS containing 1% paraformaldehyde
(Fisher) and analyzed within ne week.

Cytotoxicity Assay

5   a. <u>Target cell preparation</u>. Approximately 16-20 hours
prior to the CTL assay, target cells (Class I matched
EBV-transformed lines) were washed once and resuspended in a
10 ml volume at 3×10^5/ml in RPMI/5% FCS in the presence or
absence of 10 µg/ml total peptide.

10   b. <u>Labeling of target cells</u>: Target cells were
centrifuged and resuspended in 200 µl/tube sodium ^{51}Cr
chromate (NEN), then incubated at 37°C for 1 hour on a shaker.
Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and
resuspended in 10 ml (to determine the efficiency of
15 labelling, 50 µl/target was counted on the Micromedic
automatic gamma counter).

      c. <u>CTL assay</u>. Target cells were adjusted to 2×10^5/ml
and 50 µl of the cell culture was added to each well of a
U-bottomed 96-well plate (Costar Corp.) for a final
20 concentration of 1×10^4/well. K562 cells were washed once,
resuspended at 4×10^6/ml, and 50 µl/well was added for a
final concentration of 2×10^5/well (ratio of cold K562 to
target was 20:1). Responder cells were washed once,
resuspended at 9×10^6/ml, and three fold serial dilutions
25 were performed for effector to target ratios of 90:1, 30:1,
10:1, and 3:1. Responder cells were added in a volume of 100
µl in duplicate wells. For spontaneous release, 50 µl/well of
labelled target cells, 50 µl/well K562, and 100 µl/well of
medium was added. For maximum release, 50 µl/well target, 50
30 µl/well K562, and 100 µl/well of 0.1% Triton-X100 (Sigma) was
added. Plates were centrifuged for 5 minutes at 1200 RPM.
Following a 5 hour incubation at 37°C, plates were centrifuged
again for 5 minutes at 1200 RPM, and 100 µl/well of
supernatant was collected. Standard gamma counting techniques
35 (Micromedic automatic gamma counter; 0.5 minutes/tube) were
used t determine the percent specific lysis acc rding to the
formula: % specific lysis = cpm experimental - cpm sp ntaneous
release/cpm maximum release - cpm spontan ous release X 100.

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A cytotoxicity assay (CTL assay) was considered p sitive if th lysis by CTL f targets sensitiz d with a specific peptide at the tw highest effect r t target (E:T) ratios was 15% greater than lysis of control targets (i.e., target cells
5   without peptide). A cytotoxicity assay (CTL assay) was considered borderline if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T ratios was 6% greater than lysis of control targets (i.e., target cells without peptide).
10   d.   Results. Of the peptides that bind to the indicated alleles, 9 of the 49 MAGE peptides, 10 of the 45 HIV peptides, 3 of the 25 HCV peptides, and 2 of the 20 HBV peptides tested to date induced primary CTL in vitro. Representative graphs illustrating CTL responses to various immunogenic peptides are
15   shown for MAGE (Figure 22), HIV (Figure 23), HCV (Figure 24), and HBV (Figure 2). The CTL induction data are summarized in Table 24 which lists the immunogenic peptides which bind to the appropriate MHC and induce primary CTL in vitro. Indicated is the peptide's sequence, corresponding antigen and
20   HLA allele to which it binds. Results shown in Figure 20 illustrate lysis of peptide sensitized targets and endogenous targets following stiumlation with SAC-I activated PBMCs loaded with a MAGE 3 peptide, 1044.07 by the cold temperature and incubation technique. Figure 21 shows a comparison of the
25   acid strip loading technique (Panel a) with the cold temperature incubation technique (panel b).

        Although the present invention has been described in some detail by way of illustration and example for purposes of
30   clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

TABLE 13

VALIDATION OF CYTEL'S HLA MOTIFS

Binding Capacity (IC_{50} nM)						
Sequence	Motif	A1	A2.1	A3.2	A11	A24
AADKAAAAY	A1	50	--*	--	--	--
ATAKAAAAY	A1	15	--	329	77	--
ATDKAAAAY	A1	2.8	--	9250	840	ND
ALAKAAAAV	A2.1	--	125	--	--	--
AMAAAAAAK	A3.2	--	--	48	8.4	--
ATAAAAAAK	A11	--	--	59	40	--
AYAKAAAAF	A24	--	--	--	--	115

*A dash indicates an IC_{50} greater than 20,000 nM.

TABLE 14

VALIDATION OF CYTEL'S HLA MOTIFS

Binding Capacity (IC_{50} nM)					
SEQUENCE	MOTIF	A1	A2.1	A3.2	A11
AADKAAAAAY	A1	45	--*	--	--
ATAKAAAAAY	A1	58	--	1100	1030
ATDKAAAAAY	A1	4.0	--	10000	4533
ALAKAAAAAV	A2.1	ND	1400	--	--
AMAAAAAAAK	A3.2	ND	--	85	24.0
ATAAAAAAAK	A11	--	--	216	88

*A dash indicates an IC_{50} greater than 20,000 nM.

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TABLE 15

HLA-A3.2

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
952.25	ALAAAAAAK	1	-
952.26	AMAAAAAK	1.2	position 2
952.23	AVAAAAAK	0.95	
981.04	ASAAAAAK	0.89	
952.24	AIAAAAAK	0.57	
952.27	AAAAAAAK	0.57	
981.06	ATAAAAAK	0.49	
981.08	AFAAAAAK	0.13	
981.09	AGAAAAAK	0.077	
981.13	ACAAAAAK	0.031	
981.12	ADAAAAAK	0.014	
981.11	ANAAAAAK	0.0010	
981.05	AKAAAAAK	<0.0016	
981.07	AYAAAAAK	<0.0005	
981.10	APAAAAAK	<0.0006	
952.35	ALAAAAAR	0.46	position 9
981.36	ALAAAAAY	0.15	
981.33	ALAAAAAA	0.0034	
981.35	ALAAAAAQ	<0.0006	
981.37	ALAAAAAS	<0.0005	
981.38	ALAAAAAT	<0.0005	
981.34	ALAAAAAN	<0.0005	
981.39	ALAAAAAE	<0.0003	

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TABLE 16

HLA-A11

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
952.25	ALAAAAAAK	1	-
952.26	AMAAAAAK	2.5	position 2
952.27	AAAAAAAK	1.1	
952.24	AIAAAAAK	0.72	
981.06	ATAAAAAK	0.55	
981.04	ASAAAAAK	0.46	
981.09	AGAAAAAK	0.38	
952.23	AVAAAAAK	0.23	
981.11	ANAAAAAK	0.23	
981.13	ACAAAAAK	0.019	
981.08	AFAAAAAK	0.020	
981.12	ADAAAAAK	0.012	
981.05	AKAAAAAK	0.0065	
981.07	AYAAAAAK	<0.0065	
981.10	APAAAAAK	<0.0051	
952.35	ALAAAAAR	0.015	position 9
981.33	ALAAAAAA	<0.0059	
981.34	ALAAAAAN	<0.0071	
981.35	ALAAAAAQ	<0.0051	
981.36	ALAAAAAY	<0.0071	
981.37	ALAAAAAS	<0.0051	
981.38	ALAAAAAT	<0.0051	
981.39	ALAAAAAE	<0.0071	

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TABLE 17

HLA-A24

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 983.01	SUBSTITUTION
983.01	AYAKAAAAF	1	-
983.08	AFAKAAAAF	0.24	position 2
983.09	APAKAAAAF	0.0058	
983.10	AAAKAAAAF	0.0023	
983.11	AKAKAAAAF	<0.0012	
983.05	AYAKAAAAI	0.20	position 9
983.04	AYAKAAAAL	0.11	
983.06	AYAKAAAAV	0.0023	
983.02	AYAKAAAAA	<0.0012	
983.03	AYAKAAAAY	<0.0012	
983.07	AYAKAAAAK	<0.0012	

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TABLE 18

HLA-A1

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
982.011	ATDKAAAAY	Motif	-
982.07	ATAKAAAAY	1	-
982.09	ASAKAAAAY	0.17	position 2
982.13	AMAKAAAAY	0.095	no D in pos 3
982.08	AAAKAAAAY	0.0064	
954.09	ALAKAAAAY	0.0045	
954.11	AIAKAAAAY	0.0045	
954.13	AVAKAAAAY	0.0020	
982.10	AKAKAAAAY	0.0011	
982.11	ANAKAAAAY	<0.0001	
982.12	ADAKAAAAY	<0.0001	
982.14	AGAKAAAAY	<0.0001	
982.15	APAKAAAAY	<0.0001	
982.16	AYAKAAAAY	<0.0001	
982.17	AHAKAAAAY	<0.0001	
982.24	ATAKAAAAA	0.0040	position 9
982.23	ATAKAAAAF	0.0019	no D in pos 3
982.28	ATAKAAAAH	0.0010	
982.32	ATAKAAAAV	0.0005	
982.25	ATAKAAAAN	<0.0001	
982.26	ATAKAAAAD	<0.0001	
982.27	ATAKAAAAW	<0.0001	
982.30	ATAKAAAAK	<0.0001	
982.31	ATAKAAAAI	<0.0001	
982.29	ATAKAAAAP	<0.0001	

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TABLE 19

HLA-Al

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
982.01	ATDKAAAAY	Motif	-
982.07	ATAKAAAAY	1	-
982.01	AADKAAAAY	0.14	position 3
954.03	AAEKAAAAY	0.038	no T in pos 2
982.02	AAAKAAAAY	0.0055	
982.06	AASKAAAAY	0.0024	
982.04	AANKAAAAY	0.0011	
982.03	AAQKAAAAY	0.0008	
982.05	AAKKAAAAY	<0.0001	
982.20	AADKAAAAA	0.0016	position 9
982.21	AADKAAAAW	0.0005	no T in pos 2
982.19	AADKAAAAF	<0.0001	
982.22	AADKAAAAK	<0.0001	

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TABLE 20(A)

HPV16 E6 AND E7 PEPTIDES BINDING TO HLA-A1

Orgin	First aa Position	Sequence[*]	Binding Ratio to Standard[‡]	Motif Prediction
E6	80	ISEYRHYAY	3.500	+
E6	69	VADKALKFY	0.240	+
E7	44	QAEPDRAHY	0.029	+
E7	37	EIDGPAGQA	0.025	-
E7	19	TTDLYAYEQ	0.023	+
E6	144	MSAARSSRT	0.019	+/-
E7	73	HVDIRTLED	0.014	-
E6	139	WTGRAMSAA	0.010	-
E6	61	YRDGNPYAV	0.008	-

[*] Bold A's indicate residues in which cysteine was replaced by alanine.

[‡] The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 81±30 nM. Listed in the table are peptides yielding ratio values of ≥0.001. All other peptides yielded ratio values of ≤0.001.

TABLE 20(B)

HPV16 E6 and E7 Peptides Binding to HLA-A3.2

Origin	First aa Position	Sequence*	Binding Ratio to Standard‡	Motif Prediction
E6	107	LIRAINAQK	3.7000	+
E6	59	IVYRDGNPY	3.0000	+
E7	89	IVAPIASQK	2.2000	+
E6	33	IILEAVYAK	1.5000	+
E6	125	HLDKKQRFH	0.4400	+
E6	143	AMSAARSSR	0.1800	+
E6	7	AMFQDPQER	0.1000	+
E6	93	TTLEQQYNK	0.0780	+
E6	37	AVYAKQQLL	0.0320	–
E7	51	HYNIVTFAA	0.0210	–
E6	145	SAARSSRTR	0.0200	+
E6	75	KFYSKISEY	0.0100	+
E6	89	SLYGTTLEQ	0.0080	–
E7	52	YNIVTFAAK	0.0067	–
E6	80	ISEYRHYAY	0.0064	+
E6	42	QQLLRREVY	0.0058	–
E6	68	AVADKALKF	0.0056	+
E6	97	QQYNKPLAD	0.0045	–
E6	79	KISEYRHYA	0.0044	–
E6	84	RHYAYSLYG	0.0036	–
E6	69	VADKALKFY	0.0025	+
E6	146	AARSSRTRR	0.0020	+
E7	58	AAKADSTLR	0.0016	+
E6	38	VYAKQQLLR	0.0012	–
E6	67	YAVADKALK	0.0012	+
E7	60	KADSTLRLA	0.0012	–

* Bold A's indicate residues in which cysteine was replaced by alanine.

‡ The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 30±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

Table 20(C)

HPV16 E6 and E7 Peptides Binding to HLA-A11.2

Orgin	First aa Position	Sequence[*]	Binding Ratio to Standard[‡]	Motif Prediction
E6	33	IILEAVYAK	6.7000	+
E6	93	TTLEQQYNK	1.8000	+
E7	89	IVAPIASQK	1.3000	+
E6	7	AMFQDPQER	0.8400	+/-
E6	59	IVYRDGNPY	0.4700	- (+)[§]
E6	80	ISEYRHYAY	0.4300	- (+)[§]
E6	37	AVYAKQQLL	0.0450	-
E6	145	SAARSSRTR	0.0330	+/-
E6	107	LIRAINAQK	0.0120	+
E7	58	AAKADSTLR	0.0110	+/-
E6	42	QQLLRREVY	0.0084	+/- (+)[§]
E6	143	AMSAARSSR	0.0084	-
E6	79	KISEYRHYA	0.0076	-
E6	67	YAVADKALK	0.0074	+
E7	52	YNIVTFAAK	0.0060	+
E6	68	AVADKALKF	0.0037	-
E6	69	VADKALKFY	0.0030	- (+)[§]
E6	38	VYAKQQLLR	0.0022	+/-
E6	140	TGRAMSAAR	0.0012	+/-
E7	90	VAPIASQKP	0.0012	-
E7	51	HYNIVTFAA	0.0010	-

[*] Bold A's indicate residues in which cysteine was replaced by alanine.

[‡] The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 10±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

[§] Brackets indicate score according to adjusted motif.

Table 20(D)

HPV16 E6 and E7 Peptides Binding to HLA-A24

Orgin	First aa Position	Sequence[*]	Binding Ratio to Standard[‡]	Motif Prediction
E6	87	AYSLYGTTL	0.1200	+
E6	72	KALKFYSKI	0.1100	- (+)[§]
E6	131	RFHNIRGRW	0.1000	+
E7	49	RAHYNIVTF	0.0670	- (+)[§]
E6	49	VYDFAFRDL	0.0610	+
E6	82	EYRHYAYSL	0.0460	+
E6	26	LQTTIHDII	0.0200	-
E6	66	PYAVADKAL	0.0055	-
E6	1	MHQKRTAMF	0.0049	-
E6	85	HYAYSLYGT	0.0037	-
E6	44	LLRREVYDF	0.0023	+
E6	38	VYAKQQLLR	0.0011	-

[*] Bold A's indicate residues in which cysteine was replaced by alanine.

[‡] The average IC_{50} value ±SE of the standard in the course of the experiments considered in this table was 22±6 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

[§] Brackets indicate score according to adjusted motif.

TABLE 20(B)

SUMMARY OF EFFICACY OF 2, (3), 9 ANCHOR MOTIFS

Percentage Binders with Motifs	HLA Allele						
	A1		A3.2		A11.2		A24
	Predicted/Found		Predicted/Found		Predicted/Found		Predicted/Found
High (≥0.1)	2	2(100%)	7	7(100%)	6	6(100%)	2	3(67%)
Intermediate (0.1-0.01)	3	6(50%)	3	5(60%)	3	4(75%)	2	4(50%)
Weak (0.01-0.001)	0	1(0%)	6	14(43%)	6	11(55%)	1	5(20%)
Negative (≤0.001)	7	231(3%)	16	214(7%)	14	219(6%)	2	228(1%)
Totals	12	240	32	240	26	240	7	240

TABLE 21(A)

A3.2 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
IK	3(15%)	4(20%)	7(35%)	6(30%)	20(100%)
IR	3(15.8%)	4(21.1%)	7(36.8%)	5(26.3%)	19(100%)
LK	13(48.1%)	6(22.2%)	5(18.5%)	3(11.1%)	27(100%)
LR	4(8.5%)	9(19.1%)	20(42.6%)	14(29.8%)	47(100%)
SK	0	0	0	0	0
SR	0	0	0	1(100%)	1(100%)
TK	3(15%)	6(30%)	7(35%)	4(20%)	20(100%)
TR	2(16.7%)	1(6.0%)	2(16.7%)	7(58.3%)	12(100%)
VK	9(28.1%)	13(40.6%)	9(28.1%)	1(3.1%)	32(100%)
VR	2(7.4%)	3(11.1%)	14(51.9%)	8(29.6%)	27(100%)
Total	39(19%)	46(22.4%)	71(34.6%)	49(23.9%)	205(100%)

TABLE 21(B)
All 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
GK	0	1(100%)	0	0	1(100%)
IK	5(25%)	5(25%)	7(35%)	3(15%)	20(100%)
LK	6(22.2%)	10(37%)	9(33.3%)	2(7.4%)	27(100%)
TK	10(50%)	4(20%)	4(20%)	2(10%)	20(100%)
VK	12(37.5%)	15(46.9%)	4(12.5%)	1(3.1%)	32(100%)
Total	33(33%)	35(35%)	24(24%)	8(8%)	100(100%)

TABLE 22(A)
A3.2 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
AR	0	0	1(100%)	0	1(100%)
FK	0	0	0	0	0
FR	0	0	1(25%)	3(75%)	4(100%)
IK	0	6(27.3%)	10(45.5%)	6(27.3%)	22(100%)
IR	1(7.1%)	1(7.1%)	2(14.2%)	10(71.4%)	14(100%)
LK	16(53.3%)	7(23.3%)	5(16.7%)	2(6.7%)	30(100%)
LR	4(12.5%)	9(28.1%)	11(34.3%)	8(25%)	32(100%)
MK	1(100%)	0	0	0	1(100%)
MR	1(100%)	0	0	0	1(100%)
TK	2(11.8%)	5(29.4%)	8(47.1%)	2(11.8%)	17(100%)
TR	1(4.8%)	1(4.8%)	9(42.9%)	10(47.6%)	21(100%)
VK	7(35%)	4(20%)	5(25%)	4(20%)	20(100%)
VR	0	6(21.4%)	15(53.6%)	7(25%)	28(100%)
Total	34(17.5%)	40(20.6%)	68(35.1%)	52(26.8%)	194(100%)

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TABLE 22(B)
All 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
CK	0	0	1(100%)	0	1(100%)
GK	0	0	0	0	0
IK	4(18.2%)	5(22.7%)	12(54.5%)	1(4.5%)	22(100%)
LK	9(30%)	12(40%)	8(26.7%)	1(3.3%)	30(100%)
MK	1(100%)	0	0	0	1(100%)
TK	6(35.3%)	5(29.4%)	0	0	17(100%)
VK	7(35%)	8(40%)	5(29.4%)	1(5.9%)	20(100%)
			4(20%)	1(5%)	
Total	28/29.8%	31/33%	31/33%	4/4.3%	94/100%

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0000	HLDMLRHLY	9	c-ERB2			62	1	9.1	0.057	0.0002	
1.0046	LLDIDETEY	9	c-ERB2			849	1	7.6	0.0003	0	
1.0005	GTQLFEDNY	9	c-ERB2			104	1	0.18	0	0.029	
1.0055	LTCSPQPEY	9	c-ERB2			1131	1	0.13	0	0.0051	
1.0017	ETLEEITCY	9	c-ERB2			401	1	0.043			
1.0749	PTHQSDVWSY	10	c-ERB2			899	1	2.7	0.0003	0.0005	
1.0747	RLLDIDETEY	10	c-ERB2			848	1	1.3	0.0017	0	
1.0715	TLEEITCYLY	10	c-ERB2			402	1	1.1	0	0	
1.0737	YVMAGVGSPY	10	c-ERB2			772	1	1.1	0.010	0.012	0
1.0764	GTPTAENPEY	10	c-ERB2			1239	1	0.063			
1.0705	LIQRNPQLCY	10	c-ERB2			154	1	0.030			
1.0680	VVQGNLELTY	10	c-ERB2			55	1	0.018			
1.0756	MGDLVDAEEY	10	c-ERB2			1014	1	0.012			
1.1028	KIRKYTMRR	9	c-ERB2			651	3.11		0.76	0.0018	
1.1027	VVFGILIKR	9	c-ERB2			649	3.11		0.11	0.72	
1.1044	LVKSPNHVK	9	c-ERB2			852	3.11		0.48	0.070	
1.1035	VLRENTSPK	9	c-ERB2			754	3.11		0.40	0.013	
1.1029	ILIKRRQQK	9	c-ERB2			673	3.11		0.38	0.0097	
1.1011	ILWKDIFHK	9	c-ERB2			167	3.11		0.28	0.31	
1.1033	KITDFGLAR	9	c-ERB2			840	3.11		0.17	0.24	
1.1069	GVVFGILIK	9	c-ERB2			648	3.11		0.0007	0.089	
1.1059	QVCTGTDMK	9	c-ERB2			24	3.11		0.0007	0.052	
1.1091	LLDHVRENR	9	c-ERB2			806	3.11		0.057	<0.0006	
1.1026	CVNCSQFLR	9	c-ERB2			528	3.11		0.0015	0.001	
1.1023	TVCAGGCAR	9	c-ERB2			218	3.11		0.0004	0.023	
1.1031	ILCSTELRK	9	c-ERB2			714	3.11		0.019	0.0023	
1.1024	VTAEDGTQR	9	c-ERB2			322	3.11		<0.0002	0.014	
1.0326	DLSYMPIWK	9	c-ERB2			607	3.11		0.0005	0.010	
1.0707	TILWKDIFHK	10	c-ERB2			166	3.11		0.043	3.6	
1.0712	GTQRCEKCSK	10	c-ERB2			327	3.11		0.021	0.61	
1.0736	KVLRENTSPK	10	c-ERB2			753	3.11		0.38	0.72	
1.0702	QLISLTEILK	10	c-ERB2			143	3.11		0.20	0.013	
1.1142	RLVHRDLAAR	10	c-ERB2			840	3.11		0.18	0	
1.0741	LLNWCMQIAK	10	c-ERB2			822	3.11		0.14	0.14	
1.0752	TIDVYMIMVK	10	c-ERB2			948	3.11		0.013	0.12	
1.0731	RILKETELRK	10	c-ERB2			713	3.11		0.057	0.11	
1.0745	VLVKSPNHVK	10	c-ERB2			851	3.11		0.012	0.0072	
1.1131	SVPQNLQVIR	10	c-ERB2			423	3.11		0.017	0.075	
1.1133	HTVPWDQLFR	10	c-ERB2			470	3.11		0.0005	0.072	
1.1127	ILKCGVLIQR	10	c-ERB2			148	3.11		0.040	0.0005	
1.1143	LVSEFSRMAR	10	c-ERB2			972	3.11		0.0072	0.033	
1.1136	GVVFGILIKR	10	c-ERB2			648	3.11		0.018	0.033	
1.0726	CVARCFSGVK	10	c-ERB2			596	3.11		0.022	0.0042	
1.1137	VVFGILIKRR	10	c-ERB2			649	3.11		0.0030	0.016	
1.0728	GILIKRRQQK	10	c-ERB2			672	3.11		0.015	0.0014	
1.1129	RTVCAGGCAR	10	c-ERB2			217	3.11		0.0048	0.013	
1.1134	GLACHQLCAR	10	c-ERB2			508	3.11		0.011	0	
1.1139	KIPVAIKVLR	10	c-ERB2			747	3.11		0.0009	0.0099	

Table 23(a)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0291	VCEADYFEY	9	EBNA1			409	1	0.016			
1.0295	PLRESIVCY	9	EBNA1			553	1	0.010			
1.0681	PVCEADYFEY	10	EBNA1			408	1	0.015			
1.0683	GTWVAGVPVY	10	EBNA1			501	1	0.014			
1.0293	GVFVYGGSK	9	EBNA1			506	3.11		0.30	0.61	
1.1016	KTSLYNLRR	9	EBNA1			514	3.11		0.31	0.12	
1.0297	AIKDLVMTK	9	EBNA1			570	3.11		0.048	0.004	
1.0687	QTHIFAEVLK	10	EBNA1			567	3.11		0.010	0.21	
1.1124	CTALAIPQCR	10	EBNA1			523	3.11		0.0028	0.056	

Table 23(b)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
5.0005	CTELKLSDY	9	FLU	A	NP	44	1	3.6			
5.0006	STLELRSRY	9	FLU	A	NP	377	1	0.020			
5.0044	ILRGSVAHK	9	FLU	A	NP	265	3		1.5	0.0007	
5.0051	RMCNILKGK	9	FLU	A	NP	221	3		0.27	0.082	
5.0046	LMQGSTLPR	9	FLU	A	NP	166	3		0.031	0.10	
5.0048	MIDGIGRFY	9	FLU	A	NP	32	3		0.059	0.0010	
5.0049	MVLSAFDER	9	FLU	A	NP	66	3		0.0016	0.041	
5.0054	YIQMCTELK	9	FLU	A	NP	40	3		0.0001	0.000	
5.0042	GINDRNFWR	9	FLU	A	NP	200	3		0.0028	0.024	
5.0104	SLMQGSTLPR	10	FLU	A	NP	165	3		0.12	0.84	
5.0095	KMIDGIGRFY	10	FLU	A	NP	31	3		0.50	0.0079	
5.0096	LILRGSVAHK	10	FLU	A	NP	264	3		0.36	0.037	
5.0102	RSGAAGAAVK	10	FLU	A	NP	175	3		0.019	0.0046	
5.0105	SSTLELRSRY	10	FLU	A	NP	376	3		0.0018	0.016	
5.0103	RSRYWAIRTR	10	FLU	A	NP	382	3		0.012	0	
5.0101	RMVLSAFDER	10	FLU	A	NP	65	3		0.0014	0.010	
5.0061	FYIQMCTEL	9	FLU	A	NP	39	24				2.9
5.0060	AYERMCNIL	9	FLU	A	NP	218	24				0.031
5.0112	RFYIQMCTEL	10	FLU	A	NP	38	24				0.15

Table 23(c)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0155	LLDTASALY	9	HBV	adr	CORE	420	1	25	0.0007	0	
1.0186	SLDVSAAFY	9	HBV	adr	POL	1001	1	17.2	0.0007	0.0006	
2.0125	PTTGRTSLY	9	HBV	ALL		1,382	1	1.3	0.0008	0	
2.0126	MSTTDLEAY	9	HBV	adr		1,521	1	0.85	<0.0008	0	
1.0208	PTTGRTSLY	9	HBV	adr	POL	1382	1	0.77	0	0	
1.0387	LTKQYLNLY	9	HBV	adw	POL	1280	1	0.50	0.0000	0.0075	
2.0122	LTKQYLNLY	9	HBV	adw		1,280	1	0.095			
1.0166	KVCNPTGLY	9	HBV	adr	POL	629	1	0.068			
2.0127	MSPTDLEAY	9	HBV	adw		1,550	1	0.067			
2.0120	PSQPSRGNY	9	HBV	ayw		984	1	0.057			
2.0112	PSSWAFAKY	9	HBV	adw		316	1	0.054			
2.0119	QSAVRKEAY	9	HBV	adw		881	1	0.025			
1.0174	PLDKCICPY	9	HBV	adr	POL	698	1	0.019			
1.0378	SLMLLYKTY	9	HBV	adw	POL	1092	1	0.017			
2.0115	ASRDLVVSY	9	HBV	ayw		499	1	0.013			
2.0124	PSRCRLGLY	9	HBV	adr/adw		1,364	1	0.011			
2.0121	SSTSRNINY	9	HBV	adr		1,036	1	0.0097			
1.0519	DLLDTASALY	10	HBV	adr	CORE	419	1	11.1	0	0	
1.0513	LLDPRVRGLY	10	HBV	adr	ENV	120	1	6.3	0.17	0	
2.0239	LSLDVSAAFY	10	HBV	ALL		1,000	1	4.2	<0.0009	0.0007	
1.0911	FLCQQYLHLY	10	HBV	adr	POL	1250	1	1.1	0.014	0.0048	0.0017
2.0216	QTFCRKLHLY	10	HBV	ayw	POL	1087	1	1.1	0.0056	0.012	
2.0244	KTYGRKLHLY	10	HBV	adw		1,088	1	0.60	0.59	0.22	0
1.0791	KTYGRKLHLY	10	HBV	adw	POL	1088	1	0.57	0.53	0.36	0.0001
2.0242	QTFCRKLHLY	10	HBV	ayw		1,087	1	0.37	0.0007	0.011	
1.0556	KTFCRKLHLY	10	HBV	adr	POL	1089	1	0.34	0.094	0.080	0
2.0241	KTFCRKLHLY	10	HBV	adr		1,089	1	0.30	0.15	0.085	0
1.0766	LQDPRVRALY	10	HBV	adw	ENV	120	1	0.21	0.014	0	
1.0806	TTPAQGTSMY	10	HBV	adw	ENV	288	1	0.20	0	0	
2.0240	LSSTSRNINY	10	HBV	adr		1,035	1	0.20	<0.0009	0	
1.0541	PLDKCICPYY	10	HBV	adr	POL	698	1	0.16	0	0	
2.0238	HSASFCGSPY	10	HBV	ayw		767	1	0.15	0.019	0.017	0
1.0795	FLTKQYLNLY	10	HBV	adw	POL	1279	1	0.12	0	0	
2.0237	RSASFCGSPY	10	HBV	adr/adw		738	1	0.11	0.003	0.020	0
1.0774	WLWGMDIDPY	10	HBV	adw	CORE	416	1	0.081			
2.0233	TTPAQGTSMY	10	HBV	ayw		288	1	0.066			
1.0542	HTLWKAGILY	10	HBV	adr	POL	723	1	0.030			
2.0231	TSCPPICPGY	10	HBV	adr		226	1	0.018			
2.0246	KSVQHLESLY	10	HBV	adw		1,161	1	0.016			
1.0910	NLYVSLLLLY	10	HBV	adr	POL	1059	1	0.015			
1.0901	WMQWYWGPSL	10	HBV	adr	ENV	359	2.1	0.0009	0.019	0	0.0005
2.0089	LLYQTFGRK	9	HBV	ayw	POL	1084	3		1.3	0.44	
2.0116	IMPARFYPK	9	HBV	ayw		713	3		0.99	1.5	
2.0082	CLHQSPVRK	9	HBV	ayw	POL	867	3		0.14	0.025	
5.0056	SAKCSVVRR	9	HBV		POL	531	3		<0.0003	0.067	
2.0077	HLHQDIIKK	9	HBV	ayw	POL	686	3		0.041	0.0073	
2.0219	SLPQEHIIQK	10	HBV	ayw	POL	1197	3		0.36	4.2	
2.0234	SMFPSCCCTK	10	HBV	adr/adw		295	3		0.43	1.9	
2.0215	SMYPSCCCTK	10	HBV	ayw		295	3		1.1	1.79	
5.0107	QAFTPSPTYK	10	HBV		POL	646	3		0.15	1.3	
2.0214	LLLYQTFGRK	10	HBV	ayw	POL	1083	3		0.89	0.021	
2.0245	YMDDVVLGAK	10	HBV	ALL		1,123	3		0.16	0.0076	
5.0108	TSAKCSVVRR	10	HBV		POL	530	3		0.0006	0.013	
2.0094	PTYKAFLCK	9	HBV	ayw	POL	1243	11		0.030	0.085	
2.0068	PTDLEAYFK	9	HBV	adw	X	1552	11		0.0002	0.016	
2.0061	KYTSFPWLL	9	HBV	ALL		1,330	24				3.6
2.0059	LYAAVTNFL	9	HBV	adw		1,169	24				3.2
2.0046	FYPNLTKYL	9	HBV	adr		649	24				2.1
2.0045	LYSSTVPSF	9	HBV	adw/ayw		665	24				1.9
2.0048	FYPKVTKYL	9	HBV	ayw		718	24				1.7
2.0049	FYPNVTKYL	9	HBV	adw		718	24				1.6
2.0039	LYSILSPFL	9	HBV	ayw		368	24				0.50
2.0044	LYSSTVPVL	9	HBV	adr		636	24				0.37
2.0038	LYNILSPFL	9	HBV	adr		368	24				0.34
2.0051	NYRVSWPKF	9	HBV	ayw		991	24				0.18
2.0050	HYFQTRHYL	9	HBV	adw/ayw		743	24				0.15
2.0047	HYFKTRHYL	9	HBV	adr		714	24				0.057
2.0060	GYPALMPLY	9	HBV	ALL		1,224	24				0.049
5.0062	AYRPPNAPI	9	HBV		NUCXNUCFUS	131	24				0.026
2.0054	LYQTFGRKL	9	HBV	ayw		1,085	24				0.014
2.0043	SYQHFRRLL	9	HBV	ayw		607	24				0.011
2.0181	LYSHPIILGF	10	HBV	ALL		1,077	24				1.1
2.0182	LYAAVTNFLL	10	HBV	adw		1,169	24				0.32
2.0188	LYRPLLSLPF	10	HBV	adr		1,371	24				0.25

Table 23(d)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0174	SYQHFRRLLL	10	HBV	ayw		607	24				0.16
2.0173	SYQHFRKLLL	10	HBV	adr/adw		578	24				0.066
2.0176	YYFEHLVNHY	10	HBV	ayw		735	24				0.040
2.0172	AYRPPNAPIL	10	HBV	ALL		521	24				0.022
2.0171	GYRWMCLRRF	10	HBV	ALL		234	24				0.011
5.0115	NFLLSLGIHL	10	HBV		POL	572	24				0.0099
1.0377	YVSLMLLYK	9	HBV	adw	POL	1090	3.11		0.31	7.4	
1.0189	LLYKTFCRK	9	HBV	adr	POL	1066	3.11		5.0	0.30	
1.0379	LLYKTYCRK	9	HBV	adw	POL	1095	3.11		2.5	0.40	
1.0370	VTKYLPLDK	9	HBV	adw	POL	722	3.11		0.014	1.3	
1.0176	RHYLHTLWK	9	HBV	adr	POL	719	3.11		1.2	0.010	
1.0067	STVPSFNFK	9	HBV	adw	POL	668	3.11		0.021	0.93	
1.0215	TTDLEAYFK	9	HBV	adr	X	1523	3.11		0.0006	0.92	
1.0848	YVSLLLLYK	9	HBV	adr	POL	1063	3.11		0.39	0.92	
1.0383	PTYKAFLTK	9	HBV	adw	POL	1274	3.11		0.17	0.71	
1.0987	HLYPVARQR	9	HBV	adr	POL	1257	3.11		0.54	0.0020	
1.0058	STNRQLGRK	9	HBV	adw	ENV	85	3.11		0.51	0.34	
1.0991	ALRFTSARR	9	HBV	adr	X	1488	3.11		0.44	<0.0005	
1.0197	FVNRPDWK	9	HBV	adr	POL	1197	3.11		0.080	0.41	
1.0060	TVNENRRLK	9	HBV	adw	POL	709	3.11		0.016	0.40	
1.1041	VVNHYFQTR	9	HBV	adw	POL	740	3.11		0.000	0.33	
1.0152	STTSTGPCK	9	HBV	adr	ENV	277	3.11		0.011	0.29	
1.0213	QVLPKLLHK	9	HBV	adr	X	1505	3.11		0.10	0.28	
1.0172	LTKYLPLDK	9	HBV	adr	POL	680	3.11		0.0009	0.23	
1.0374	CLHQSAVRK	9	HBV	adw	POL	678	3.11		0.22	0.017	
1.0980	VVDPSQFSR	9	HBV	adr	POL	943	3.11		0.011	0.20	
1.0382	FLYACIQAK	9	HBV	adw	POL	1259	3.11		0.18	0.034	
2.0074	YVNTNMGLK	9	HBV	ayw	CORE	507	3.11		0.16	0.048	
1.0199	FLYACIQSK	9	HBV	adr	POL	1230	3.11		0.11	0.018	
1.0972	RLADEGLNR	9	HBV	adr	POL	601	3.11		0.10	0.025	
1.0976	AVNHYFKTR	9	HBV	adr	POL	711	3.11		0.0071	0.088	
1.0975	RLKLIMPAR	9	HBV	adr	POL	680	3.11		0.095	0.0002	
1.0977	ILYKRETTR	9	HBV	adr	POL	730	3.11		0.095	<0.0005	
1.0993	KVFVLGGCR	9	HBV	adr	X	1548	3.11		0.082	0.082	
1.0165	NVSIPWTHK	9	HBV	adr	POL	621	3.11		0.072	0.076	
1.0982	LLLYKTFCR	9	HBV	adr	POL	1065	3.11		0.072	0.0045	
1.0978	RLVFQTSTR	9	HBV	adr	POL	757	3.11		0.066	0.0002	
1.0219	FVLGGCRHK	9	HBV	adr	X	1550	3.11		0.065	0.039	
1.1042	RLVLQTSTR	9	HBV	adw	POL	786	3.11		0.064	0.0002	
1.1043	MLLYKTYCR	9	HBV	adw	POL	1094	3.11		0.061	0.00002	
1.0170	TVNEKRRLK	9	HBV	adr	POL	674	3.11		0.048	0.007	
1.1045	NLYPVARQR	9	HBV	adw	POL	1286	3.11		0.042	0.0011	
1.1046	LPYRPTTGR	9	HBV	adw	POL	1407	3.11		0.021	0	
1.0045	LVSFGVWIR	9	HBV	adr	CORE	509	3.11		0.0033	0.020	
1.0981	LVGSSGLPR	9	HBV	adr	POL	1022	3.11		0.0008	0.015	
1.0967	HISCLTFGR	9	HBV	adr	CORE	494	3.11		0.013	0.011	
1.1047	SVPSRLFQR	9	HBV	adw	POL	1424	3.11		0.0007	0.010	
1.0969	SVPSHLFQR	9	HBV	adr	POL	1395	3.11		0.0004	0.010	
1.0564	TLPQEHIVLK	10	HBV	adr	POL	1179	3.11		0.092	5.6	
2.0205	TVPVFNPHWK	10	HBV	ayw	POL	660	3.11		0.0067	4.2	
1.0543	TLWKAGILYK	10	HBV	adr	POL	724	3.11		3.5	1.0	
1.0807	SMYPSCCCTK	10	HBV	ayw	ENV	285	3.11		1.5	3.4	
1.1153	RLPYRPTTGR	10	HBV	adw	POL	1406	3.11		2.3	0.030	
1.0584	STTDLEAYFK	10	HBV	adr	X	1522	3.11		0.0066	2.7	
1.0554	LLLYKTFCRK	10	HBV	adr	POL	1065	3.11		2.5	0.012	
1.0799	TVNAHRNLPK	10	HBV	adw	X	1529	3.11		0.82	0.66	
1.0586	EAYFKDCLFK	10	HBV	adr	X	1527	3.11		0.037	0.74	
1.1051	LVVDPSQFSR	10	HBV	adr	POL	942	3.11		0.0009	0.63	
1.0789	MLLYKTYCRK	10	HBV	adw	POL	1094	3.11		0.61	0.020	
1.0546	TAYSHLSTSK	10	HBV	adr	POL	858	3.11		0.26	0.092	
1.0562	SLGIHLNPNK	10	HBV	adr	POL	1150	3.11		0.20	0.078	
1.1152	RLGLYRPLLR	10	HBV	adw	POL	1397	3.11		0.19	0.0049	
1.0647	VTGGVFLVDK	10	HBV	adr	POL	943	3.11		0.035	0.17	
1.1150	RLRTPKTPAR	10	HBV	adw	POL	942	3.11		0.17	0.0002	
1.0561	TVNGHQVLFK	10	HBV	adr	X	1500	3.11		0.073	0.092	
1.1091	SLPFQPTTGR	10	HBV	adr	POL	1377	3.11		0.077	0.043	
1.1072	TLPETTVVRR	10	HBV	adr	CORE	532	3.11		<0.0003	0.075	
1.1089	GTDNSVVLSR	10	HBV	adr	POL	1320	3.11		0.025	0.072	
1.1071	STLPETTVVR	10	HBV	adr	CORE	531	3.11		0.0005	0.068	
2.0210	KVTKYLPLDK	10	HBV	ayw	POL	721	3.11		0.027	0.053	
1.1148	STRHGDKSFR	10	HBV	adw	POL	792	3.11		0.0057	0.038	
1.0935	VLSCWWLQFR	10	HBV	adw	POL	923	3.11		0.029	0.0087	
1.0781	NVTKYLPLDK	10	HBV	adw	POL	721	3.11		<0.0004	0.023	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.1092	RVCCQLDPAR	10	HBV	adr	X	1022	3.11		0.0019	0.023	
1.0793	SLCIHLNPQK	10	HBV	adw	POL	1179	3.11		0.017	0.014	
1.0909	YLVSFGVWIR	10	HBV	adr	CORE	508	3.11		0.015	0.0027	
2.0207	FVGPLTVNEK	10	HBV	ayw	POL	648	3.11		0.0057	0.015	
1.0535	YVGPLTVNEK	10	HBV	adr	POL	648	3.11		0.0069	0.014	
1.1075	RLADEGLNRR	10	HBV	adr	POL	601	3.11		0.013	0.0004	
1.1086	IVLKLKQCFR	10	HBV	adr	POL	1185	3.11		0.013	0.0024	
1.0773	PIFSSWAFAK	10	HBV	adw	ENV	314	3.11		<0.0003	0.010	
1.0778	LTVNENRRLK	10	HBV	adw	POL	702	3.11		0.0025	0.0095	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0118	CTCGSSDLY	9	HCV		LORF	1123	1	3.0	0	0.010	
1.0112	NIVDVQYLY	9	HCV		NS1/ENV2	697	1	0.40	0	0.010	
2.0004	VQDCNCSIY	9	HCV			302	1	0.54	0.0005	0.0003	
2.0035	LTPRCMVDY	9	HCV			605	1	0.078			
1.0145	RVCEKMALY	9	HCV		LORF	2588	1	0.053			
1.0140	DVVCCSMSY	9	HCV		LORF	2416	1	0.039			
2.0006	FTIFKIRMY	9	HCV			636	1	0.012			
1.0509	GLSAFSLHSY	10	HCV		LORF	2188	1	0.41	0.013	0.0004	0.0002
1.0499	TLHGPTPLLY	10	HCV		LORF	1617	1	0.30	0.11	0.0026	
2.0037	EYVLLLFLL	9	HCV			719	24				1.4
2.0169	MYVCGVEHRL	10	HCV			633	24				0.026
2.0170	EYVLLLFLLL	10	HCV			719	24				0.010
1.0139	SVPAEILRK	9	HCV		LORF	2269	3.11		0.016	0.87	
1.0565	QLFTFSPRR	9	HCV		ENV1	290	3.11		0.75	0.003	
1.0090	RLGVRATRK	9	HCV		CORE	43	3.11		0.74	0.16	
1.0123	LIFCHSKKK	9	HCV		LORF	1391	3.11		0.54	0.19	
1.0122	HLIFCHSKK	9	HCV		LORF	1390	3.11		0.25	0.010	
1.0952	KTSERSQPR	9	HCV		CORE	51	3.11		0.16	0.064	
1.0120	AVCTRGVAK	9	HCV		LORF	1183	3.11		0.016	0.038	
1.0143	EVFCVQPEK	9	HCV		LORF	2543	3.11		0.0019	0.083	
1.0137	ITRVESENK	9	HCV		LORF	2241	3.11		0.015	0.0079	
1.0957	CITSLTGR	9	HCV		LORF	1082	3.11		0.0095	0.011	
1.0486	GVAGALVAFK	10	HCV		LORF	1858	3.11		0.87	1.1	
1.0480	HLHAPTGSCK	10	HCV		LORF	1227	3.11		0.57	0.0051	
1.1052	RMYVCGVEHR	10	HCV		NS1/ENV2	632	3.11		0.27	0.012	
1.0485	HLIFCHSKKK	10	HCV		LORF	1390	3.11		0.27	0.025	
1.0484	TLGPGAYMSK	10	HCV		LORF	1261	3.11		0.17	0.13	
1.1067	CVGIYLLPNR	10	HCV		LORF	3002	3.11		0.0029	0.032	
1.1053	LLFLLLADAR	10	HCV		NS1/ENV2	723	3.11		0.015	0	

Table 23(e)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0014	FRDYVDRFY	9	HIV		GAG	298	1	0.090			
2.0129	IYQYMDDLY	9	HIV			875	1	0.064			
1.0029	TVLDVGDAY	9	HIV		POL	802	1	0.018			
1.0412	VTVLDVGDAY	10	HIV		POL	801	1	0.28	0	0.0004	
1.0415	VIYQYMDDLY	10	HIV		POL	874	1	0.25	0.0007	0.0090	
2.0252	VTVLDVGDAY	10	HIV			801	1	0.088			
1.0431	EVNIVTDSQY	10	HIV		POL	1187	1	0.053			
1.0441	LVAVHVASGY	10	HIV		POL	1329	1	0.009			
1.0442	PAETGQETAY	10	HIV		POL	1345	1	0.013			
2.0251	ISKIGPENPY	10	HIV			702	1	0.013			
2.0255	QMAVFIHNFK	10	HIV			1432	3		0.61	0.64	
2.0064	RYLKDQQLL	9	HIV			2778	24				0.76
2.0134	RYLKDQQLL	9	HIV			2778	24				0.32
2.0065	TYQNYQEPF	9	HIV			1033	24				0.30
2.0131	TYQNYQEPF	9	HIV			1033	24				0.20
2.0063	IYQEPFKNL	9	HIV			1036	24				0.052
2.0132	IYQEPFKNL	9	HIV			1036	24				0.003
2.0066	IYQYMDDLY	9	HIV			875	24				0.013
2.0247	IYKRWIILGL	10	HIV			266	24				0.017
2.0190	IYKRWIILGL	10	HIV			266	24				0.014
2.0249	LYFLASLESL	10	HIV			506	24				0.014
1.0049	KLAGRWPVK	9	HIV		POL	1358	3.11		2.7	0.060	
1.0044	AVFIHNFKR	9	HIV		POL	1434	3.11		0.17	1.8	
1.0032	AIFQSSMTK	9	HIV		POL	853	3.11		1.1	0.96	
1.0046	IVIWGKTPK	9	HIV		POL	1075	3.11		0.085	0.37	
1.0079	KLTEDRWNK	9	HIV		VIF	1712	3.11		0.013	0.27	
1.0007	GIPHPAGLK	9	HIV		POL	788	3.11		0.23	0.065	
1.0059	QIIEQLIKK	9	HIV		POL	1215	3.11		0.0091	0.16	
1.0309	KIWPSYKGR	9	HIV		GAG	443	3.11		0.12	0.0005	
1.0072	ILATDIQTK	9	HIV		POL	1458	3.11		0.025	0.098	
1.0006	MGYELHPDK	9	HIV		POL	925	3.11		0.064	0.096	
1.0062	YLAWVPAHK	9	HIV		POL	1227	3.11		0.077	0.057	
1.0308	KIWPSHKGR	9	HIV		GAG	443	3.11		0.077	<0.0005	
1.0047	FVNTPPLVK	9	HIV		POL	1111	3.11		0.012	0.066	
1.0024	NTPVFAIKK	9	HIV		POL	752	3.11		0.003	0.060	
1.0080	TVQCTHGIK	9	HIV		ENV	2620	3.11		0.0021	0.046	
1.0013	ILDIRQGPK	9	HIV		GAG	287	3.11		0.062	0.0048	
1.0015	RDYVDRFYK	9	HIV		GAG	299	3.11		0.0007	0.040	
1.0058	CKQAQFDK	9	HIV		POL	1199	3.11		<0.0009	0.040	
1.0064	VLFLDGIDK	9	HIV		POL	1254	3.11		0.038	0.032	
1.0026	LVDFRELNK	9	HIV		POL	769	3.11		0.011	0.030	
1.0078	KVVPRRKAK	9	HIV		POL	1513	3.11		0.029	0.0009	
1.0042	MTKILEPFR	9	HIV		POL	859	3.11		<0.0008	0.016	
1.0443	TVYYGVPVWK	10	HIV		ENV	2185	3.11		3.8	7.8	
1.0418	TVQPIVLPEK	10	HIV		POL	935	3.11		0.16	5.6	
1.0447	AVFIHNFKRK	10	HIV		POL	1434	3.11		0.46	0.85	
1.0437	KVLFLDGIDK	10	HIV		POL	1253	3.11		0.36	0.78	
1.0408	KLVDFRELNK	10	HIV		POL	768	3.11		0.51	0.090	
1.0403	KLKPGMDGPK	10	HIV		POL	706	3.11		0.39	0.076	
1.0395	FLGKIWPSYK	10	HIV		GAG	440	3.11		0.32	0.024	
1.1056	KIQNFRVYYR	10	HIV		POL	1074	3.11		0.032	0.21	
1.0410	GIPHPAGLKK	10	HIV		POL	788	3.11		0.011	0.17	
1.0406	LVKLWYQLEK	10	HIV		POL	1117	3.11		0.054	0.082	
1.0398	MIGGIGGFIK	10	HIV		POL	642	3.11		0.0099	0.055	
1.0413	MTKILEPFRK	10	HIV		POL	859	3.11		0.015	0.038	
1.0433	VVIQDNSDIK	10	HIV		POL	1504	3.11		<0.0005	0.021	
1.0394	FLGKIWPSHK	10	HIV		GAG	440	3.11		0.020	0.0013	
1.1054	IVQQQNNLLR	10	HIV		ENV	2741	3.11		0.0004	0.019	
1.0417	FTTPDKKHQK	10	HIV		POL	909	3.11		<0.0002	0.015	
1.0405	LVEICTEMEK	10	HIV		POL	729	3.11		0.0002	0.012	
1.0392	LVQNANPDCK	10	HIV		GAG	327	3.11		<0.0002	0.011	

Table 23(f)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0225	ISEYRHYCY	9	HPV	16	E6	80	1	7.3	0.0011	0.036	
1.0230	QAEPDRAHY	9	HPV	16	E7	44	1	0.021			
1.0610	LQDIEITCVY	10	HPV	18	E6	25	1	0.25	0.0056	0.012	
2.0159	YSKISEYRHY	10	HPV	16	E6	77	1	0.17	<0.0009	0	
2.0162	YSKISEYRHY	10	HPV	16	E6	77	1	0.11	<0.0009	0	
1.0599	HGDTPTLHEY	10	HPV	16	E7	2	1	0.087			
1.0601	QPETTDLYCY	10	HPV	16	E7	16	1	0.033			
1.0913	IHDIILECVY	10	HPV	16	E6	30	1	0.002			
2.0160	YSRIRELRHY	10	HPV	18	E6	72	1	0.018			
2.0164	YSRIRELRHY	10	HPV	18	E6	72	1	0.012			
1.0594	AVCDKCLKFY	10	HPV	16	E6	68	1	0.0095			
2.0161	LLIRCLRCQK	10	HPV	18	E6	101	3		0.081	0.078	
2.0002	HTMLCMCCK	9	HPV	18	E7	59	11		0.020	0.079	
2.0029	VYCKTVLEL	9	HPV	18	E6	33	24				0.33
2.0027	CYSLYGTTL	9	HPV	16	E6	87	24				0.057
2.0024	VYDFAFRDL	9	HPV	16	E6	49	24				0.002
2.0031	LYNLLIRCL	9	HPV	18	E6	98	24				0.019
2.0000	VYGDTLEKL	9	HPV	18	E6	85	24				0.010
1.0239	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.39	2.3	
1.0243	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.53	1.1	
1.0244	SVYGDTLEK	9	HPV	18	E6	84	3.11		0.70	0.95	
1.0226	TTLEQQYNK	9	HPV	16	E6	93	3.11		0.018	0.67	
1.0241	SIPHAACHK	9	HPV	18	E6	59	3.11		0.0094	0.25	
1.0237	SIPHAACHK	9	HPV	18	E6	59	3.11		0.017	0.12	
1.0233	IVCPICSQK	9	HPV	16	E7	80	3.11		0.035	0.023	
1.0997	KLRHLNEKR	9	HPV	18	E6	117	3.11		0.025	<0.0005	
1.0234	LIRCLRCQK	9	HPV	18	E6	102	3.11		0.019	0.0012	
1.0853	IILECVYCK	9	HPV	16	E6	33	3.11		0.0016	0.019	
1.0999	CIDFYSRIR	9	HPV	18	E6	68	3.11		0.017	0.0018	
1.0998	CIDFYSRIR	9	HPV	18	E6	68	3.11		0.010	0.0009	
1.0594	GTTLEQQYNK	10	HPV	16	E6	92	3.11		0.010	0.98	
1.0606	LLIRCLRCQK	10	HPV	18	E6	101	3.11		0.076	0.29	
1.0598	LLIRCINCQK	10	HPV	16	E6	105	3.11		0.12	0.34	
1.0629	LLIRCLRCQK	10	HPV	18	E6	101	3.11		0.16	0.11	
1.0614	LTEVFEFAFK	10	HPV	18	E6	41	3.11		0.0009	0.11	
1.0605	GIVCPICSQK	10	HPV	16	E7	88	3.11		0.0017	0.060	
1.0625	LTEVFEFAFK	10	HPV	18	E6	41	3.11		0.0012	0.061	
1.0591	DIILECVYCK	10	HPV	16	E6	32	3.11		0.0065	0.021	
1.1101	KLRHLNEKRR	10	HPV	18	E6	117	3.11		0.013	0	
1.1095	CVYCKQQLLR	10	HPV	16	E6	37	3.11		0.011	0.0059	

Table 23(g)

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Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0020	EVDPIGHLY	9	MAGE	3		161	1	18	0.0002	0.0009	
3.0172	EADPTSNTY	9	MAGE	5/51		161	1	9.9	0.0006	0.0006	0
1.0258	TQQLVQEKY	9	MAGE	1		240	1	2.1	0	0.0002	
3.0173	EVDPICHVY	9	MAGE	6		161	1	1.9	<0.0002	<0.0002	0
1.0254	EADPTCHSY	9	MAGE	1		161	1	1.1	0	0	
1.0259	LVQEKYLEY	9	MAGE	1		243	1	0.42	0.0013	0.053	
6.0053	TSYVKVLEY	9	MAGE	1	new	275	1	0.099			
2.0009	SSLPTTMNY	9	MAGE	3		9	1	0.053			
2.0011	CSVVGNWQY	9	MAGE	3		77	1	0.050			
2.0008	SSPSTTINY	9	MAGE	2		9	1	0.043			
1.0252	MLESVIKNY	9	MAGE	1		128	1	0.011			
2.0147	ASSLPTTMNY	10	MAGE	3		8	1	2.6	<0.0009	0.013	
2.0167	LTQQLVQEKY	10	MAGE	1		239	1	1.2	<0.0009	0.0073	
6.0114	ETSYVKVLEY	10	MAGE	1	new	274	1	0.56			
2.0141	ASSPSTTINY	10	MAGE	2		8	1	0.17	<0.0009	0.026	
1.0648	DLVQEKYLEY	10	MAGE	1		242	1	0.044			
6.0065	TSYVKVLEY	9	MAGE	1	new	275	3		0.71	0.010	
4.0119	TTINFTRQR	9	MAGE	1		66	3		0.043	0.37	
6.0064	ALAETSYVK	9	MAGE	1	new	271	3		0.31	0.36	
4.0132	LTQQLVQEK	9	MAGE	1		239	3		<0.0008	0.14	
6.0063	LVQEKYLEY	9	MAGE	1	new	243	3		0.0006	0.004	
4.0131	HSAYGEPRK	9	MAGE	1		229	3		0.014	0.0009	
4.0122	LFRAVITKK	9	MAGE	1		97	3		0.011	0.0005	
6.0124	RVRFFFPSLR	10	MAGE	1	new	290	3		0.43	0.0009	
4.0161	ADLVGFLLLK	10	MAGE	1		107	3		0.35	0.29	
4.0160	ESLFRAVITK	10	MAGE	1		95	3		0.14	0.088	
6.0119	DLVQEKYLEY	10	MAGE	1	new	242	3		0.002	0.0051	
6.0123	YVIKVSARVR	10	MAGE	1	new	283	3		0.019	0.0009	
4.0168	LSVMEVYDCR	10	MAGE	1		218	3		<0.0008	0.012	
4.0163	KAEMLESVIK	10	MAGE	1		125	3		<0.0008	0.0077	
6.0125	RALAETSYVK	10	MAGE	1	new	270	11		0.18	0.24	
2.0010	NYPLWSQSY	9	MAGE	3		16	24				0.027
2.0165	NYKHCFPEIF	10	MAGE	1		135	24				0.25
2.0151	LYIFATCLGL	10	MAGE	3		115	24				0.048
6.0126	SYVKVLEYVI	10	MAGE	1	new	276	24				0.036
1.0248	SLFRAVITK	9	MAGE	1		96	3.11		4.1	2.7	
1.1006	SVMEVYDCR	9	MAGE	1		219	3.11		0.0080	1.3	
1.1004	TTINFTRQR	9	MAGE	1		66	3.11		0.016	1.0	
1.0257	LTQQLVQEK	9	MAGE	1		239	3.11		0.0002	0.38	
1.0634	SLFRAVITKK	10	MAGE	1		96	3.11		1.2	0.98	
1.0647	LLTQQLVQEK	10	MAGE	1		238	3.11		0.0004	0.16	
1.0640	MLESVIKNYK	10	MAGE	1		128	3.11		0.14	0.027	
1.0644	LLGDNQQMPK	10	MAGE	1/3		162	3.11		0.020	0.011	
1.0630	SLEQRSLHCK	10	MAGE	1		2	3.11		0.015	0.015	

Table 23(h)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0281	CSDCTTIHY	9	pS3			226	1	29.5	0.0010	0.029	
1.0667	CTAKSVTCTY	10	pS3			117	1	0.33	0.023	0.049	0
1.0672	RVEGNLRVEY	10	pS3			196	1	0.022			
1.0278	RVRAMAIYK	9	pS3			156	3.11		1.5	0.73	
1.0276	CTYSPALNK	9	pS3			124	3.11		0.46	1.1	
1.0285	NTSSSPQPK	9	pS3			311	3.11		0.0009	0.095	
1.0284	RTEEENLRK	9	pS3			283	3.11		0.0015	0.091	
1.0287	ELNEALELK	9	pS3			343	3.11		0.020	0.0062	
1.0678	RTEEENLRKK	10	pS3			283	3.11		3.3	0.0080	
1.1113	KTYQGSYGFR	10	pS3			101	3.11		2.6	0.88	
1.1115	VVRRCPHHER	10	pS3			172	3.11		0.099	0.0017	
1.0679	NTSSSPQPKK	10	pS3			311	3.11		0.0005	0.054	
1.1121	RVCACPGRDR	10	pS3			273	3.11		0.014	0.011	
1.1116	GLAPPQHLIR	10	pS3			187	3.11		0.013	0.0006	

Table 23(i)

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Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
3.0175	KCEYFVEMY	9	PAP			322	1	3.4	<0.0002	0.0002	0
3.0174	LGEYIKKKY	9	PAP			81	1	0.78	<0.0002	0.0002	0
3.0164	ASCHLTELY	9	PAP			311	1	0.77	<0.0002	0.055	0
3.0163	ESYICHEQVY	9	PAP			95	1	0.098	<0.0002	0.0002	0
3.0237	LSELSLLSLY	10	PAP			238	1	14	0.0026	0.0004	0
3.0235	LSELSLLSLY	10	PAP			238	1	12	0.0005	0.0004	0
3.0236	LTQLGMEQHY	10	PAP			70	1	0.62	0.015	0.0024	0.0022
3.0238	KCEYPVEMYY	10	PAP			322	1	0.018			
3.0230	LVNEILNHMK	10	PAP			263	3		0.056	0.12	
3.0158	ATQIPSYKK	9	PAP			274	11		0.10	1.2	
3.0231	ETLKSEEPQK	10	PAP			170	11		<0.0004	0.014	
3.0161	LYFEKGEYF	9	PAP			318	24				2.5
3.0160	LYCESVHNF	9	PAP			213	24				0.44
3.0159	PYKDFIATL	9	PAP			183	24				0.11
3.0162	VYNGLLFPY	9	PAP			302	24				0.002
3.0232	PYASCHLTEL	10	PAP			309	24				0.024

Table 23(j)

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Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0270	ALPERPSLY	9	PSA			231	1	0.011			
2.0157	VSHSFPHPLY	10	PSA			88	1	0.15	<0.0003	0.0013	
1.0265	PLYDMSLLK	9	PSA			95	3.11		0.24	0.007	
1.0273	VVHYRKWIK	9	PSA			242	3.11		0.0072	0.003	
1.0272	YTKVVHYRK	9	PSA			239	3.11		0.0006	0.058	
1.1009	SLLKNRFLR	9	PSA			100	3.11		0.0024	0.047	
1.0260	IVGGWECEK	9	PSA			21	3.11		0.061	0.019	
1.0269	QVHPQKVTK	9	PSA			182	3.11		0.0060	0.014	
1.1112	SLYTKVVHYR	10	PSA			237	3.11		0.28	0.23	
1.0653	LTAAHCIRNK	10	PSA			57	3.11		0.14	0.083	
1.0651	RIVGGWECEK	10	PSA			20	3.11		0.046	0.087	
1.0642	KVVHYRKWIK	10	PSA			241	3.11		0.045	0.045	
1.1111	VTKFMLCAGR	10	PSA			188	3.11		0.0003	0.012	
3.0108	MLLRLSEPA	9	PSA			118	Random				

Table 23 (k)

TABLE 24: CTL EPITOPES IDENTIFIED IN PEPTIDE SCREENING.

Sequence	Antigen	Motif	Id
EVDPIGHLY	MAGE3	A01	1044.07
ASSLPTTMNY	MAGE3	A01	1044.01
EADPTGHSY	MAGE1	A01	958.01
SSLPTTMNY*	MAGE3	A01	1072.02*
GSVVGNWQY*	MAGE3	A01	1072.03*
ALAETSYVK*	MAGE1N	A03	1072.38*
SLFRAVITK	MAGE1	A11	1072.13
RALAETSYVK	MAGE1N	A11	1072.39
ESLFRAVITK	MAGE1	A11	1072.15
KVYLAWVPAHK	HIV	A3/11*	1069.42*
TVYYGVPVWK	HIV	A03	1069.43
KLAGRWPVK	HIV	A03	1069.44
KMIGGIGGFIK	HIV	A03	1069.45
AIFQSSMTK	HIV	A03	966.01
WTYQIYQEPFK	HIV	A03	1069.46
ELGKIWPSHK*	HIV	A03	1069.56*
TVYIYGVPVWK	HIV	A11	1052.03
VTVYYGVPVWK	HIV	A11	1069.47
GVAGALVAFK	HCV	A03	1073.10
CTCGSSDLY	HCV	A11	1069.62
GVAGALVAFK	HCV	A11	1052.05
LLDTASALY*	HBV	A01	1069.01*
TLWKAGILYK	HBV	A03	1069.15

* borderline positive

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Table 25 a
Peptides Synthesized
by Cytel For Loading
Onto Acid Stripped
Autologous PBMCs and
PHA Blasts

Peptide ID #	Antigen	Sequence
777.03	HBs	FLLTRILTI
924.07	HBc	FLPSDFFPSV
927.32	HBp	GLYSSTVPV
938.01	MAGE 1	EADPTGHSY
939.03	PSA	VLVHPQWVL
941.01	HBc	FLPSDYFPSV
1044.04	PAP	ILLWDPIPV
1044.05	PSA	KLQCVDLVHI
1044.06	PSA	MLLRLSEPAEL

Table 25 b

Cell Population	^{125}I-Labeled Peptide +/- Cold Peptide	CPMS +/- std. dev.	
JY acid stripped	- cold peptide	3553 ± 157	n = 3
JY acid stripped	+ cold peptide	13	n = 1
JY control	-cold peptide	370 ± 37	n = 3
JY control	+ cold peptide	50	n = 1

WHAT IS CLAIMED IS:

1.   A comp siti n comprising an immunogenic peptide
having a HLA-A3.2 binding motif, which immunogenic peptide has
5   between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:
       a first conserved residue selected from the group
consisting of L, M, I, V, S, A, T, F, C, G, D and E;
       and a second conserved residue of K, R, Y, H and F;
10   wherein the first and second conserved residues are
separated by 6 to 7 residues.

2.   The composition of claim 1, wherein the first
conserved residue is at the second position from the
15   N-terminus.

3.   A composition comprising an immunogenic peptide
having a HLA-A1 binding motif, which immunogenic peptide has
between about 9 and about 10 residues and the following
20   residues, from the N-terminus to the C-terminus:
       a first conserved residue of T, S and M; and
       a second conserved residue of D, E, A, S and T;
       a third conserved residue of Y;
       wherein the first and second conserved residues are
25   adjacent and the second and third conserved residues are
separated by 5 or 6 residues.

4.   The composition of claim 3, wherein the first
conserved residue is at the second position from the
30   N-terminus.

5.   A composition comprising an immunogenic peptide
having a HLA-A1 binding motif, which immunogenic peptide has
between about 9 and about 10 residues and the following
35   residues, from the N-terminus to the C-terminus:
       a first c nserved residue f T, S and M; and
       a second conserved residu   f Y;

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wherein the first and sec nd conserv d residues are
s parated by 6 to 7 residues.

6.   The composition of claim 5, wherein the first
conserved residue is at the second position from the
N-terminus and the second conserved residue is at the ninth or
tenth position from the N-terminus.

7.   A composition comprising an immunogenic peptide
having an HLA-A1 binding motif, which immunogenic peptide has
between 9 and about 10 residues and the following residues,
from the N-terminus to the C-terminus.
        a first conserved residue of D, E, A, S and T; and
        a second conserved residue of Y;
        wherein the first and second conserved residues are
separated by 5 to 6 residues.

8.   The composition of claim 5, wherein the first
conserved residue is at the third position from the N-terminus
and the second conserved residue is at the ninth or tenth
position from the N-terminus.

9.   A composition comprising an immunogenic peptide
having a HLA-A11 binding motif, which peptide has between
about 9 and about 10 residues and the following residues, from
the Nterminus to the C-terminus:
        a first conserved residue of L, M, I, V, A, S, T, G,
N, Q, C, F, D, E; and
        a second conserved residue of K, R, H;
        wherein the first and second conserved residues are
separated by 6 to 7 residues.

10.   The composition of claim 9, wherein the first
conserved residue is at the second position from the
N-terminus.

11.   A composition c mprising an immun genic peptide
having a HLA-A24.1 binding motif, which immunogenic peptid

has between about 9 and ab ut 10 residu s and the f llowing
residues, fr m the N-terminus to the C-terminus:

a first conserved residue of Y, F, W; and

a second conserved residue of F, I, L, W, M;

wherein the first and second conserved residues are
separated by 6 to 7 residues.

12.   The composition of claim 11, wherein the first
conserved residue is at the second position from the
N-terminus.

13.   A composition comprising an immunogenic peptide
having an HLA-A3.2 binding motif, which immunogenic peptide
has 9 or 10 residues:

a first conserved residue at the second position
selected from the group consisting of A, I, L, M, T, and V;
and a second conserved residue at the C terminal postion
selected from the group consisting of K and R.

wherein the first and second conserved residues are
separated by 6 to 7 residues.

14.   A composition comprising an immunogenic peptide
having an HLA-A11 binding motif, which immunogenic peptide has
9 or 10 residues and the following residues, from the
N-terminus to the Cterminus:

a first conserved residue at the second position from
the N terminus selected from the group consisting of A, I, L,
M, T and V; and

a second conserved residue at the C terminal position
selected from the group consisting of K;

wherein the first and second conserved residues are
separated by 6 to 7 residues.

15.   A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A3.2 binding motif, which immun genic peptide has
between about 9 and about 10 residues and the f ll wing
residues, fr m the N-terminus to the C-terminus:

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a first c nserved residue selected fr m th gr up
consisting f L, M, I, V, S, A, T, F, C, G, D and E; and
a second conserved residue of K, R and Y;
wherein the first and second conserved residues are
5   separated by 6 to 7 residues.

16.   A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A1 binding motif, which immunogenic peptide has
10   between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:
a first conserved residue of T, S and M; and
a second conserved residue of D, E, A, S and T;
a third conserved residue of Y;
15   wherein the first and second conserved residues are
separated by 1 residue and the second and third conserved
residues are separated by 5 or 6 residues.

17.   A pharmaceutical composition comprising a
20   pharmaceutically acceptable carrier and an immunogeric peptide
having a HLA-A1 binding motif, which immunogenic peptide has
between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:
a first conserved residue of T, S or M; and
25   a second conserved residue of Tyr;
wherein the first and second conserved residues are
separated by 6 to 7 residues.

18.   A pharmaceutical composition comprising a
30   pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A1 binding motif, which peptide has between about
9 and about 10 residues and the following residues, from the
N-
terminus to the C-terminus:
35   a first conserved residu of D, E, S, T; and
a second c nserved residue of Y;
wherein the first and sec nd conserved residues are
separated by 5 t 6 residues.

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19. A pharmaceutical comp sition comprising a
pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A24.1 binding motif, which peptide has

a first conserved residue of Y, F, W; and

5   a second conserved residue of F, I, L, W, or m;

wherein the first and second conserved residues are
separated by 6 to 7 residues.

20. A method of identifying an immunogenic peptide
10   comprising the following steps:

determining a binding motif for an MHC molecule
encoded by a preselected MHC Class I allele;

screening an amino acid sequence of an antigenic
protein for the presence of the binding motif;

15   selecting a sequence in the antigenic protein having
the binding motif;

preparing a test peptide of about 8 and about 11
residues comprising the selected subsequences;

determining the ability of the test peptide to bind
20   to the preselected MHC allele and induce a CTL response,
thereby identifying immunogenic peptides.

HLA-A PURIFICATION AND PEPTIDE ELUTION

Cellular Source: HLA-Antigens   a)   EBV transformed B cell
(5-10x10⁸ cell equivalents)   lines - homozygous
                                    b)   HLA-A transfectants -
                                         e.g. .221-HLA-A1
                                    c)   P815 transfectants
                                            (mouse mastocytoma)

Detergent Lysis   1% NP-40 or 1% Renex 30 plus
(10⁷ cells/ml)   protease inhibitors - 1 hr,4°C

Detergent Lysate   Centrifugation at 15,000xg,
                                           30 min.

Affinity Chromatography   mAb-Sepharose   5 mg/ml
                                              5-10 ml column

Purified HLA-A Antigen   Anticipated yields   450-900 µg

Acid Treatment   10% acetic acid, 5 min, 100°C

Peptides   YM3 filtration, 3kD cut-off

Sequence/Motif   D. Hunt - HPLC/EI-TMS
                                           Cytel - HPLC/ABI 477A

4/6/92:RK

Figure 1

Figure 2

RP-HPLC separation 1 HLA-A3 acid eluted peptides.
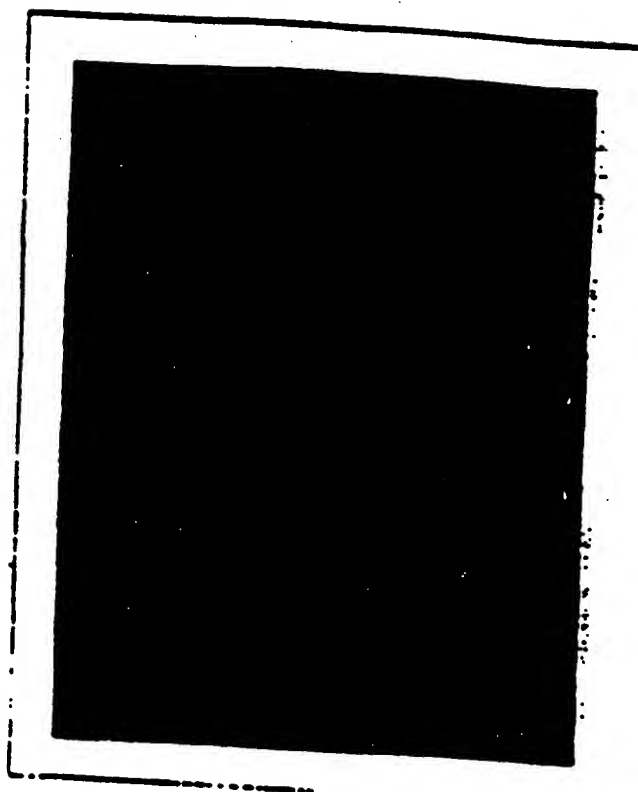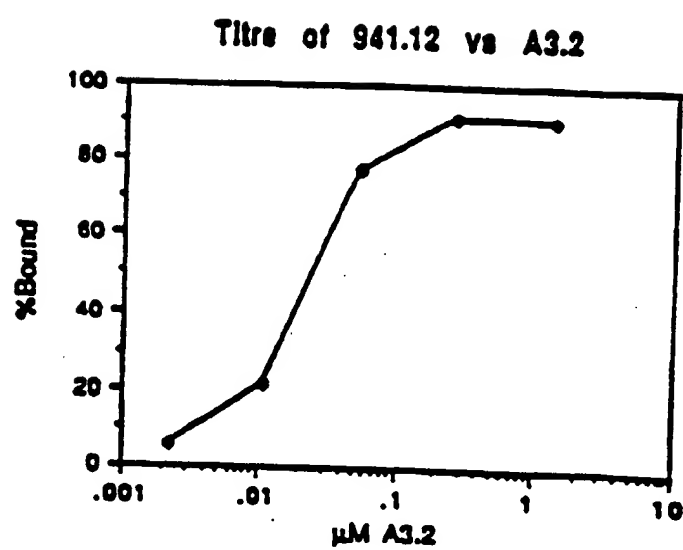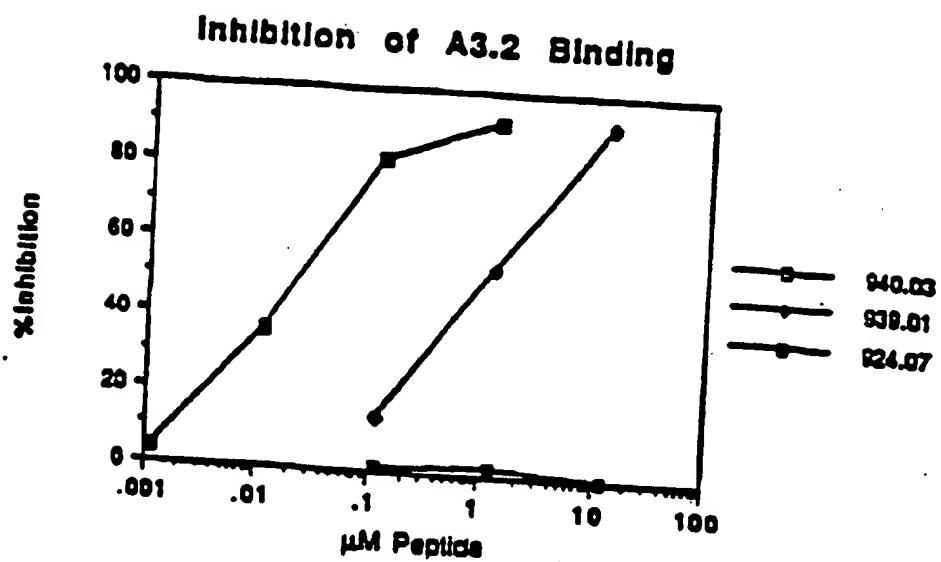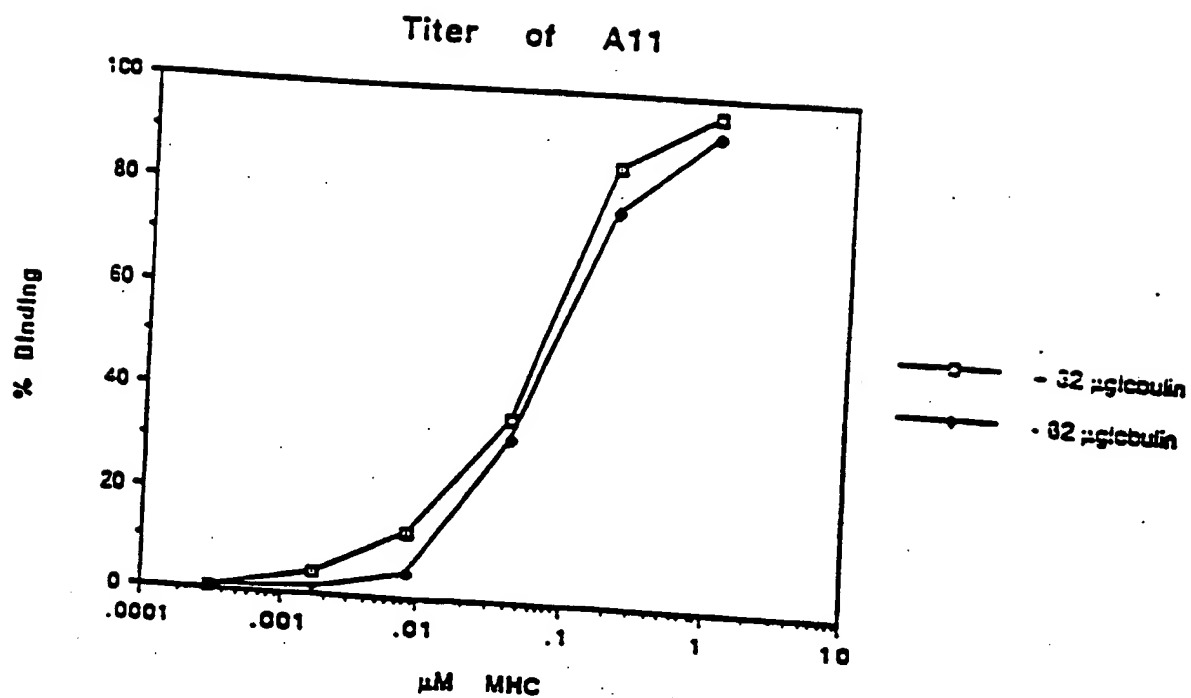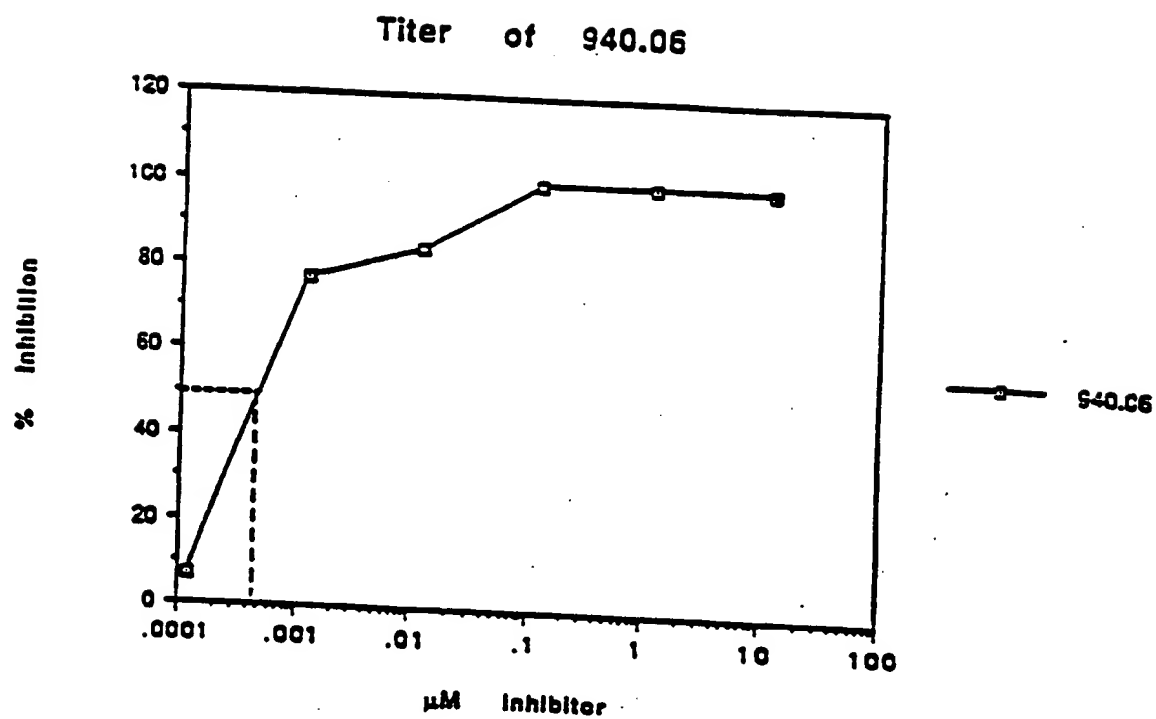
Figure 4

Figure 5
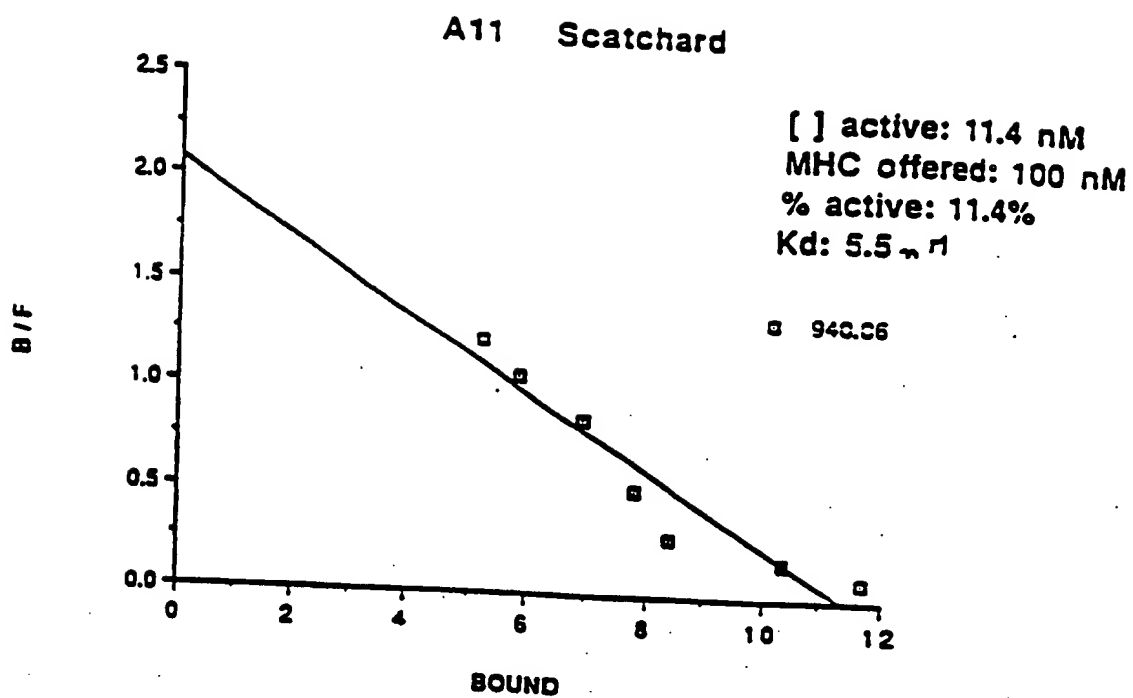
Figure 6

Figure 7

Figure 8

Titer of 944.02 against A1.
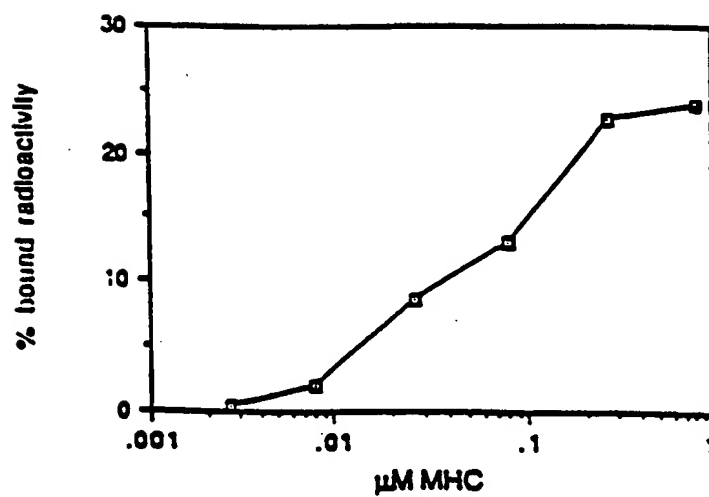
Figure 9

A1 inhibition titration.
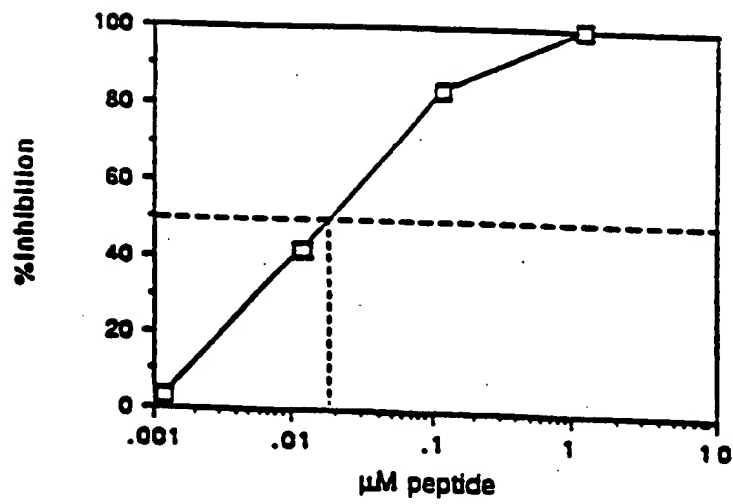
Figure 10
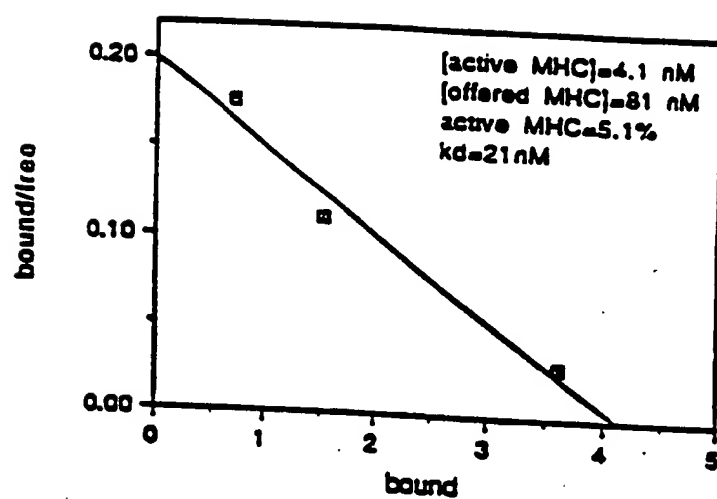
Figure 11

A24 titration.
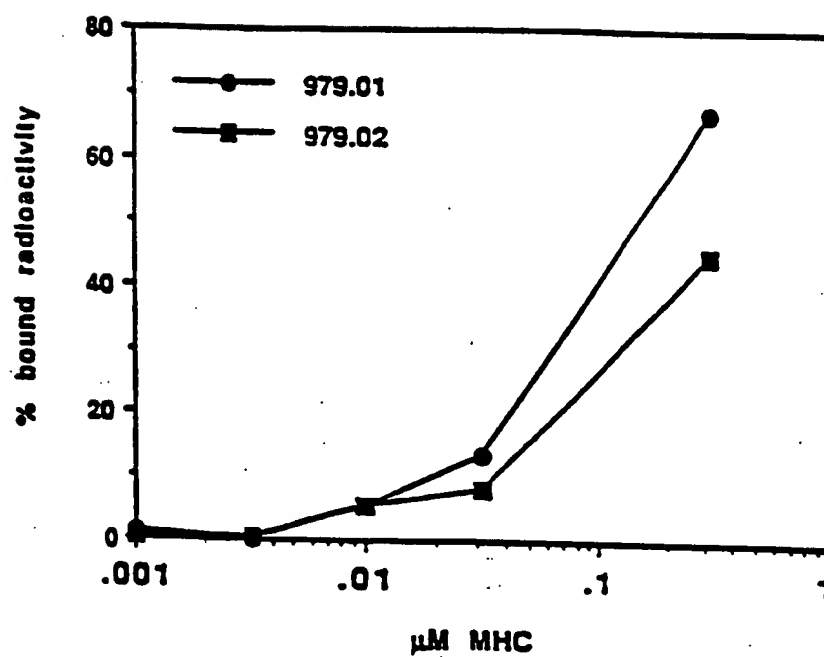
Figure 12

A24 Inhibition.

Figure 13

Figure 14A

Peptide 979.01
[active MHC]=13.6 nM
[offered MHC]=320 nM
active MHC=8.3%
kD=136 nM

Figure 14B

Peptide 979.02
[active MHC]=23.6 nM
[offered MHC]=320 nM
active MHC=7.4%
kD=28 nM
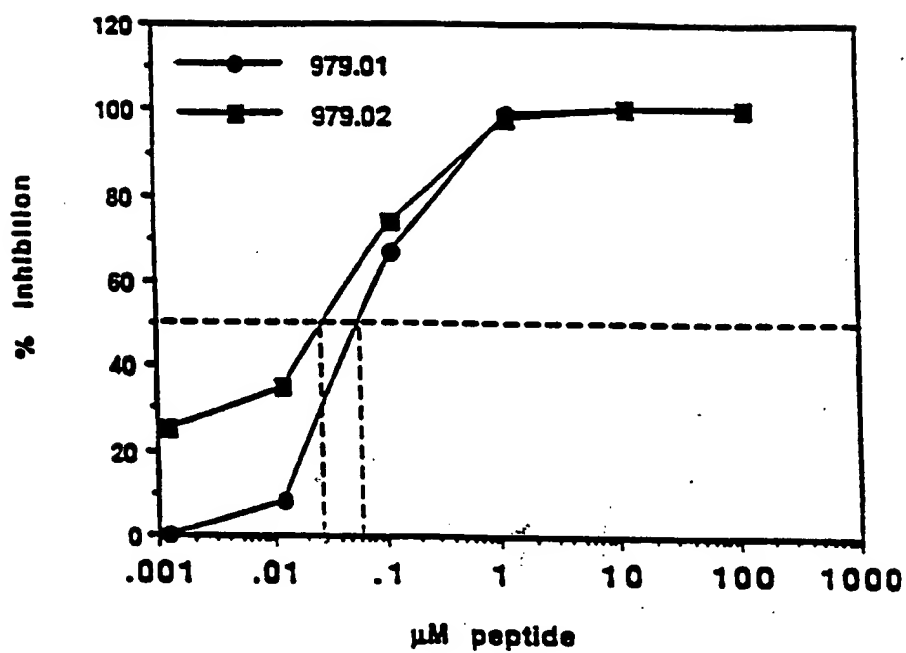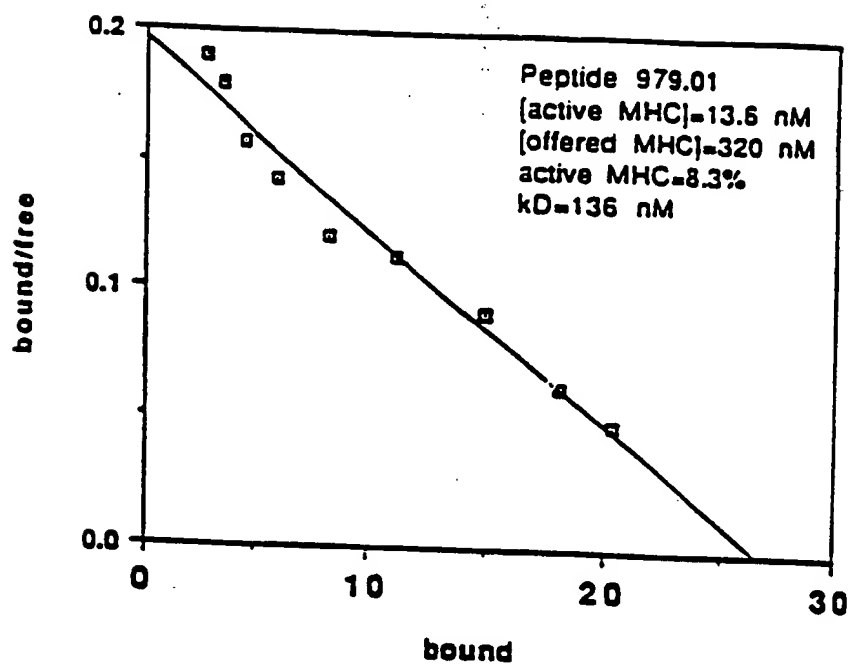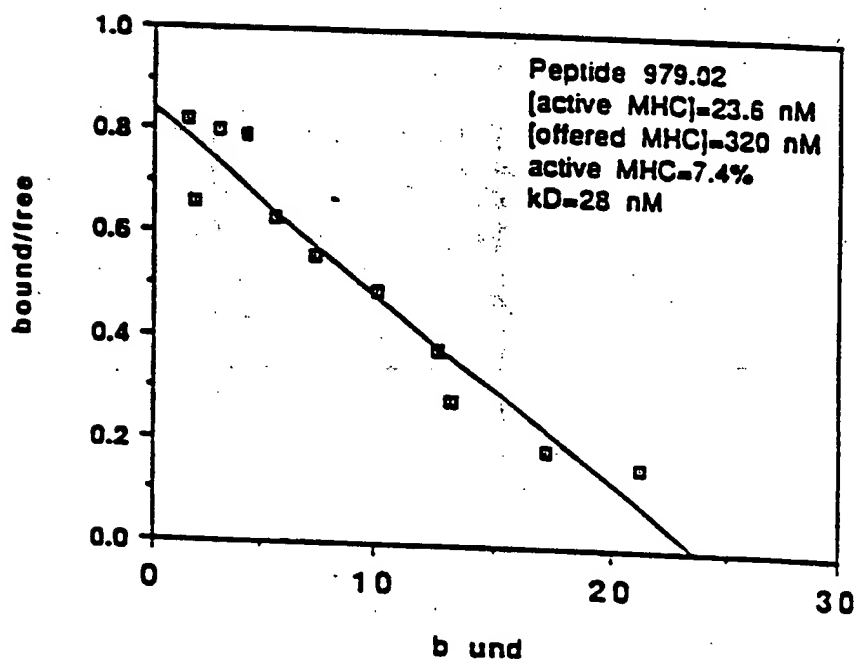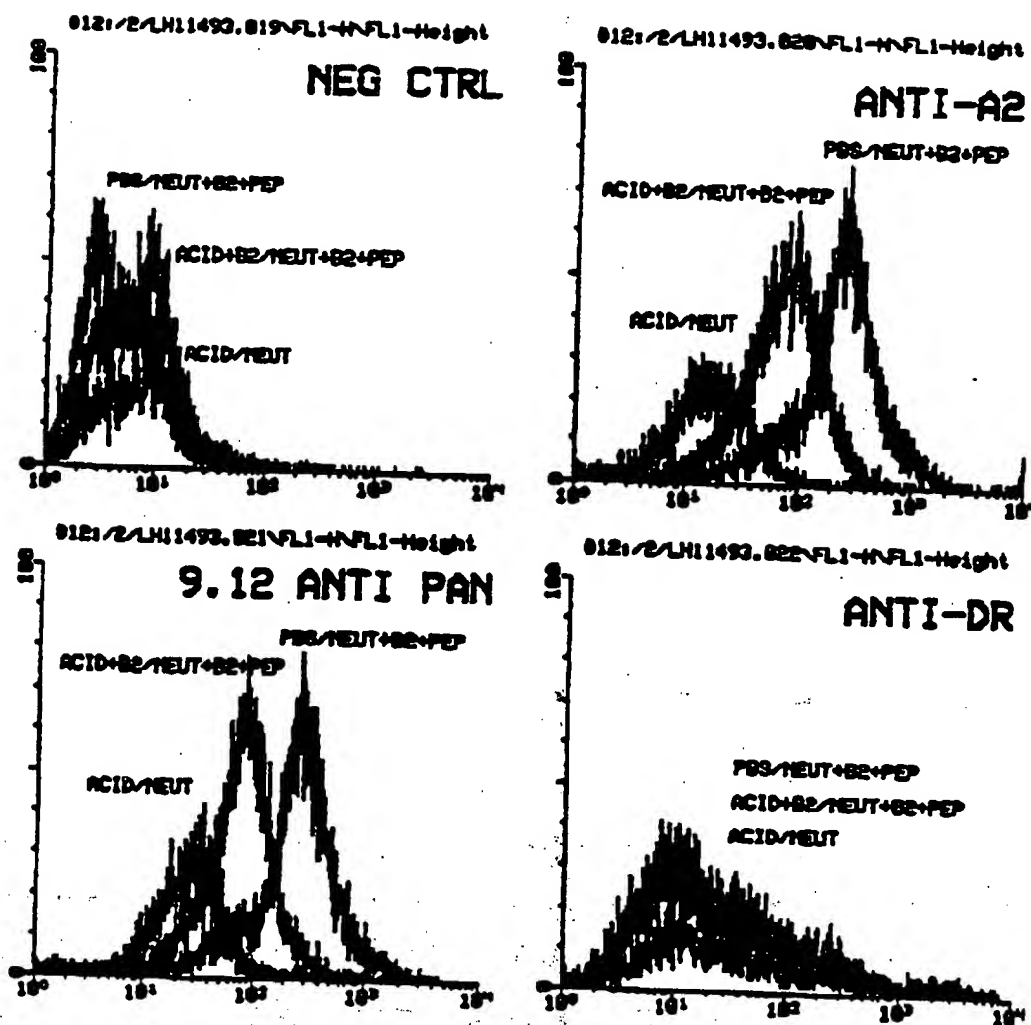
Figure 15

Figure 16

CTL INDUCTION
GC43 A2.1 CD4 DEPLETED RESPONDER
777.03 + 924.07 + 927.32 POOL

——●—— Acid Strip PBMC + peptide   ——■—— Acid Strip PHA Blasts + peptide
——○—— Acid Strip PBMC - peptide   ——□—— Acid Strip PHA Blasts - peptide

Figure 17

Figure 18

CTL INDUCTION
GC49 A2.1 CD4 DEPLETED RESPONDERS
939.03 PEPTIDE
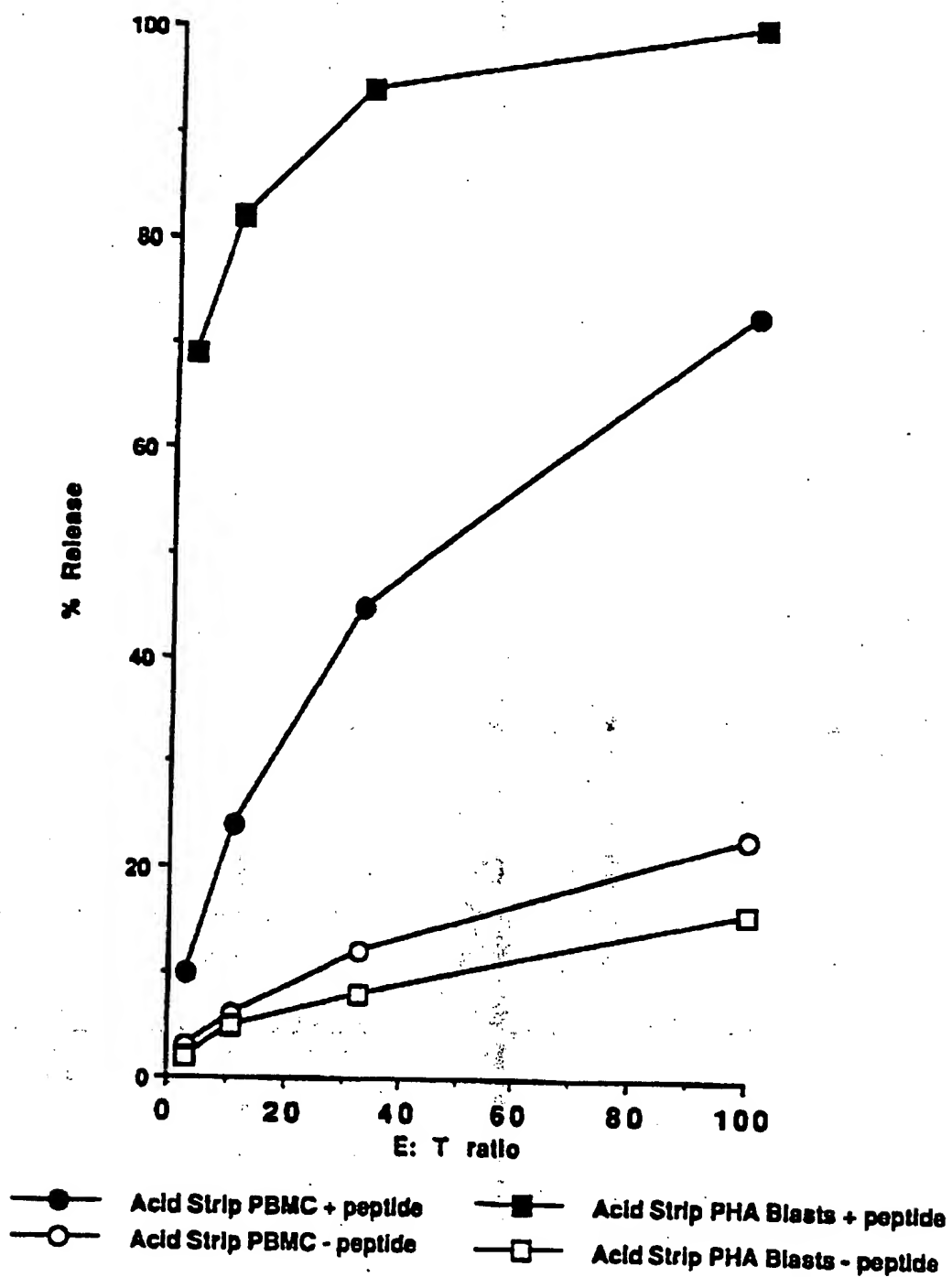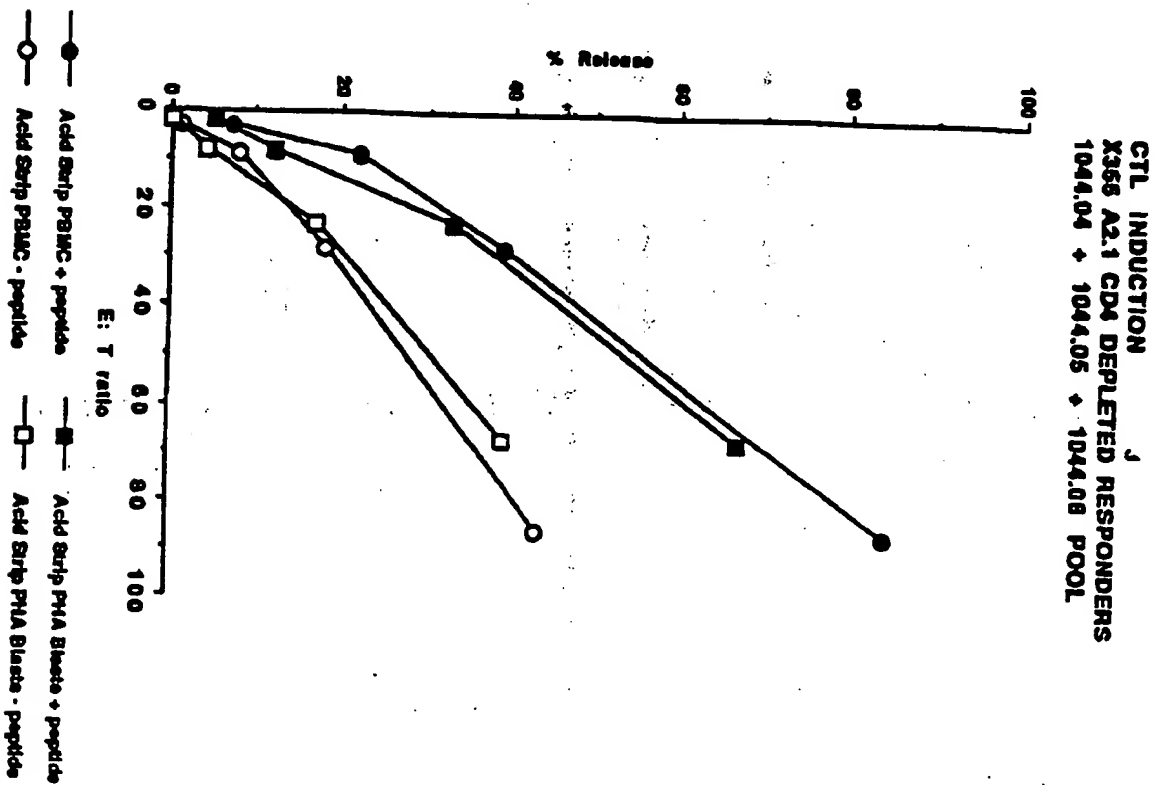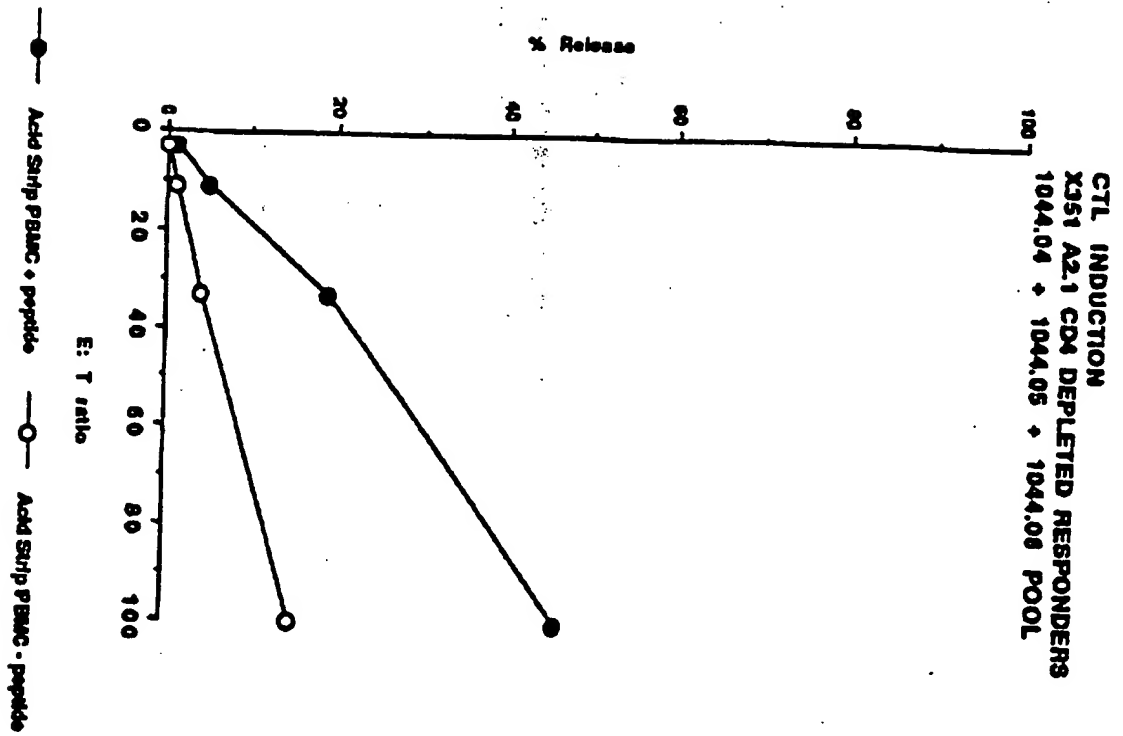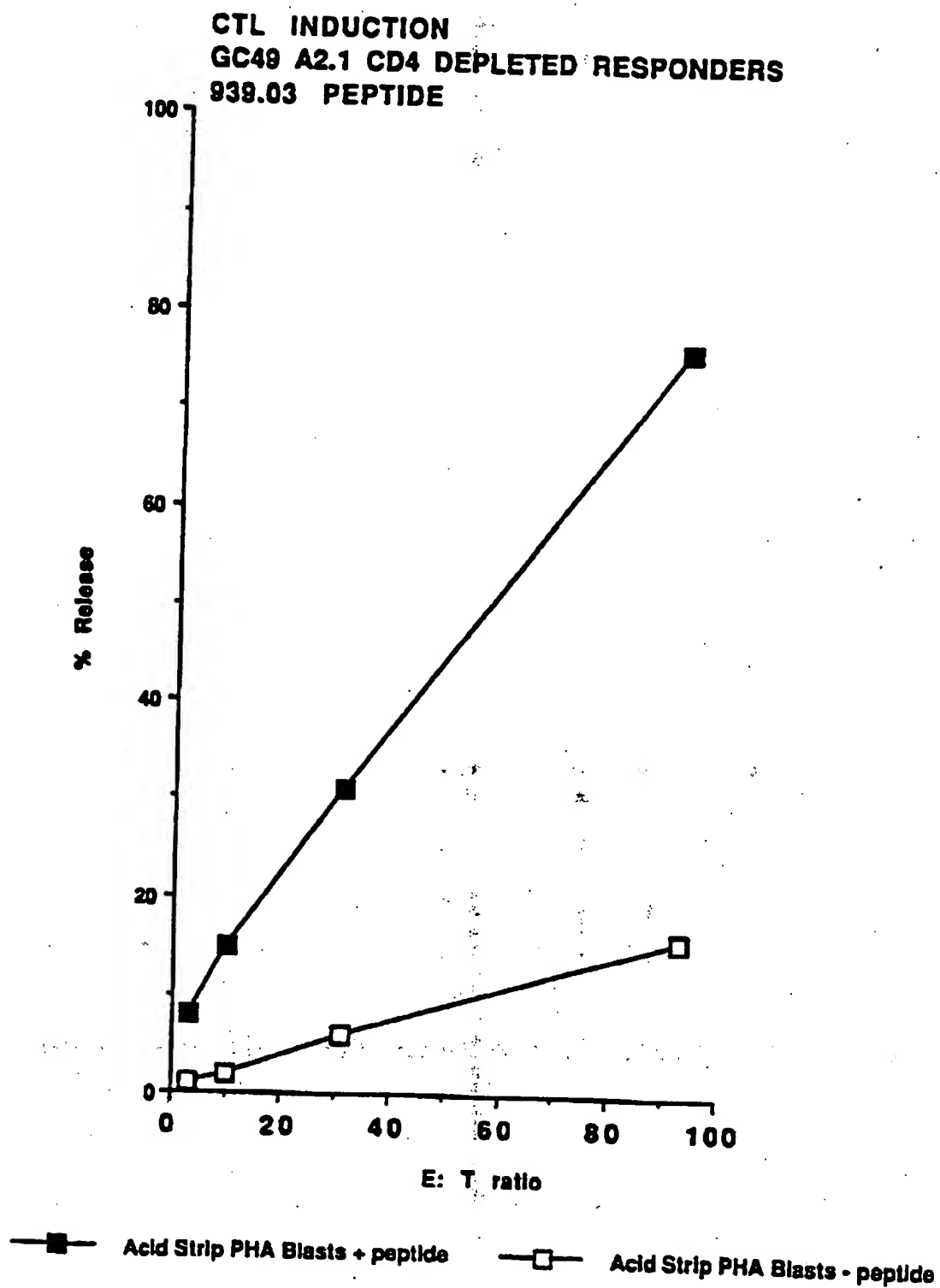
MAGE1 Peptide Specific, HLA-A1-Restricted CTL Can Kill Melanoma Cells Expressing the Endogenous Antigen
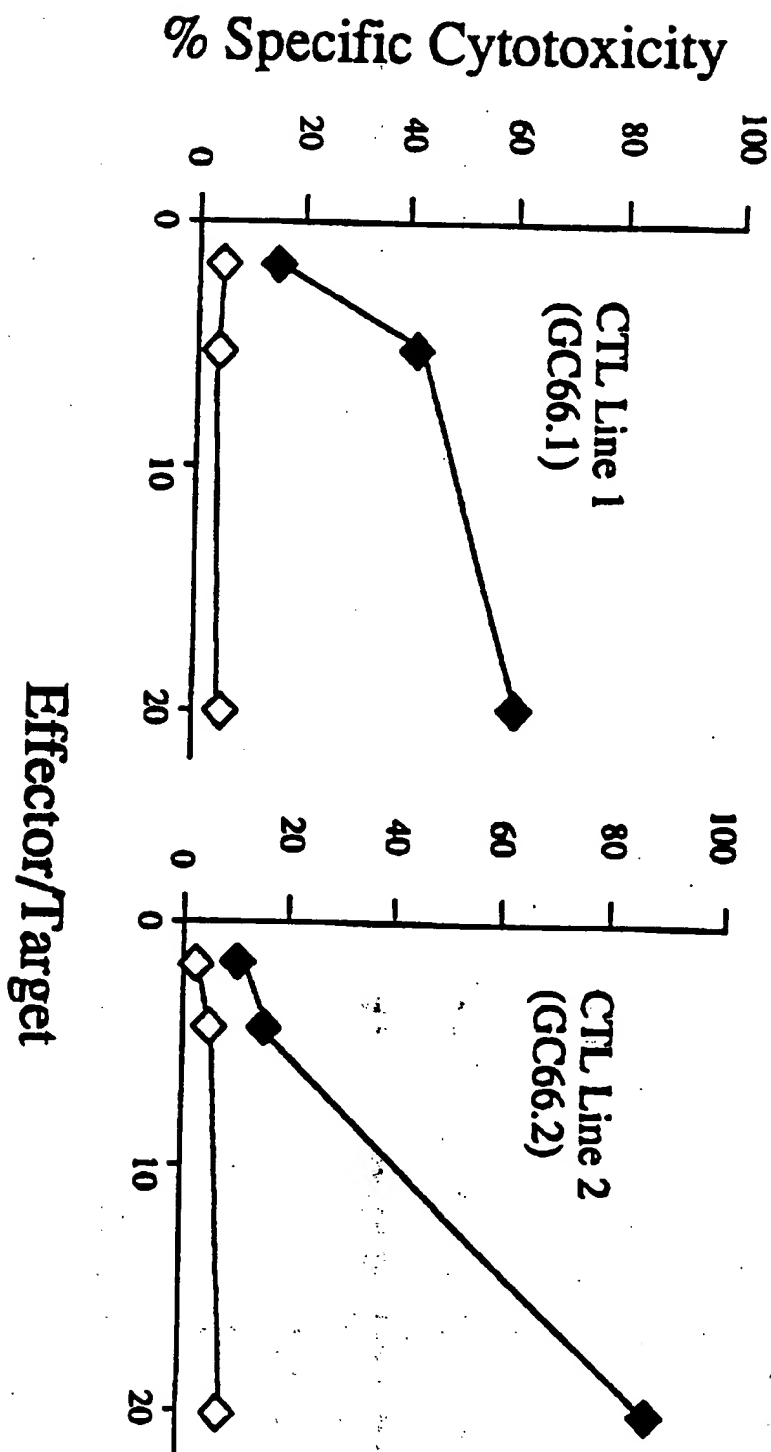
Figure 19

Figure 20

Cytotoxic Activity of an HLA-A1-Restricted CTL Line Specific for a MAGE3 Peptide (1044.07)
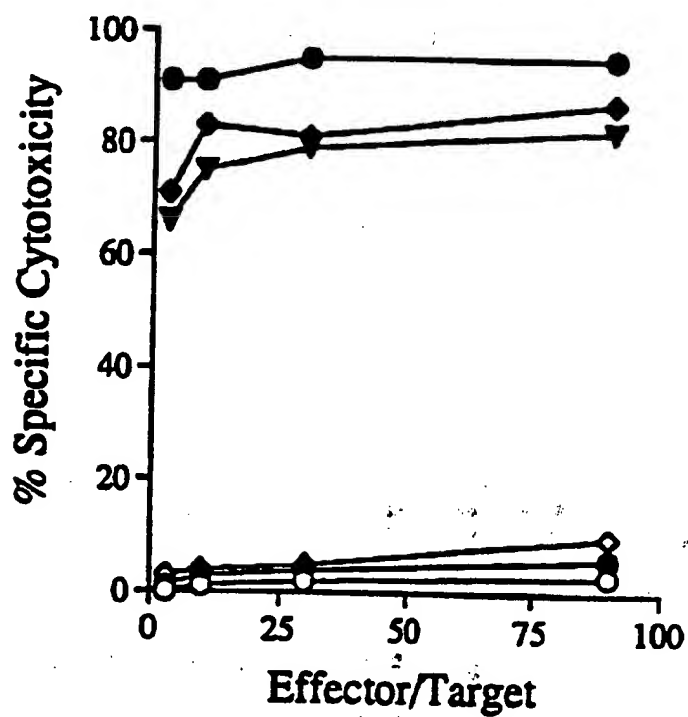
—O— Steinlin - Pep		—◇— mel 888 (MAGE-3 -)	
—●— Steinlin + 1044.01		—▼— mel 397 (MAGE-3 +)	
—●— Steinlin + 1044.07		—◆— mel 938 (MAGE-3 +)	

Fig. 21

Induction Using Different Methods
to Load Peptides nt SAC-I C lls.

Donor GC164
A1   MAGE-3   (1044.07/1044.01)
SAC-I activ. PBMCs
AS protocol

○—○ no peptide
●—● peptide pool

Steinin - K562

E:T Ratio

Donor GC164
A1   MAGE-3   (1044.07/1044.01)
SAC-I activ. PBMCs
MF protocol

○—○ no peptide
●—● peptide pool

Steinin - K562

E:T Ratio

Donor GC164
A1   MAGE-3   (1044.07/1044.01)
SAC-I activ. PBMCs
Pulse only (no MF or AS protocol)

○—○ no peptide
●—● peptide pool

Steinin - K562

E:T Ratio

Donor GC164
A1   MAGE-3   (1044.07/1044.01)
SAC-I activ. PBMCs
Dump (no MF or AS protocol)

○—○ no peptide
●—● peptide pool

Steinin - K562

Figure 22
Peptide Screening:
MAGE/A11

Donor GC210
MAGE/A11
Pool 1

% Specific Lysis

BVR + K562

E:T Ratio

- no peptide
- 1072.13
- 1072.18
- 1072.20

Donor GC210
MAGE/A11
Pool 3

% Specific Lysis

BVR + K562

E:T Ratio
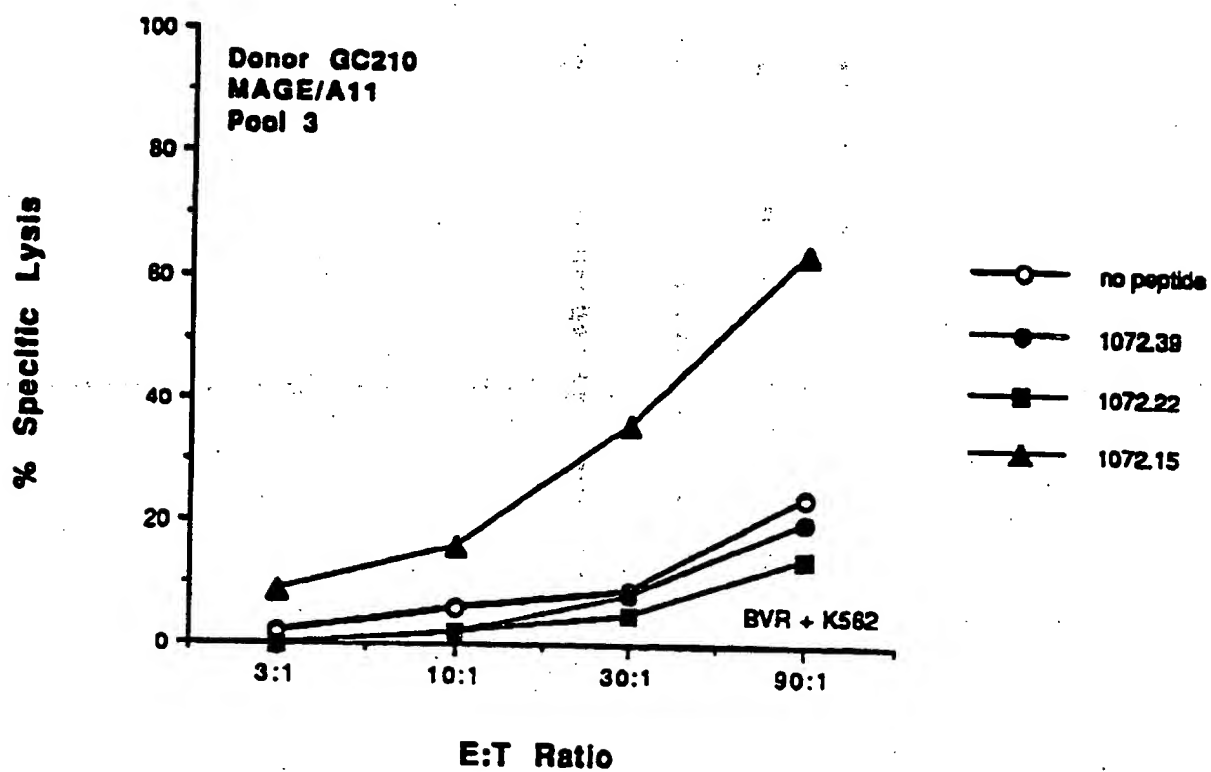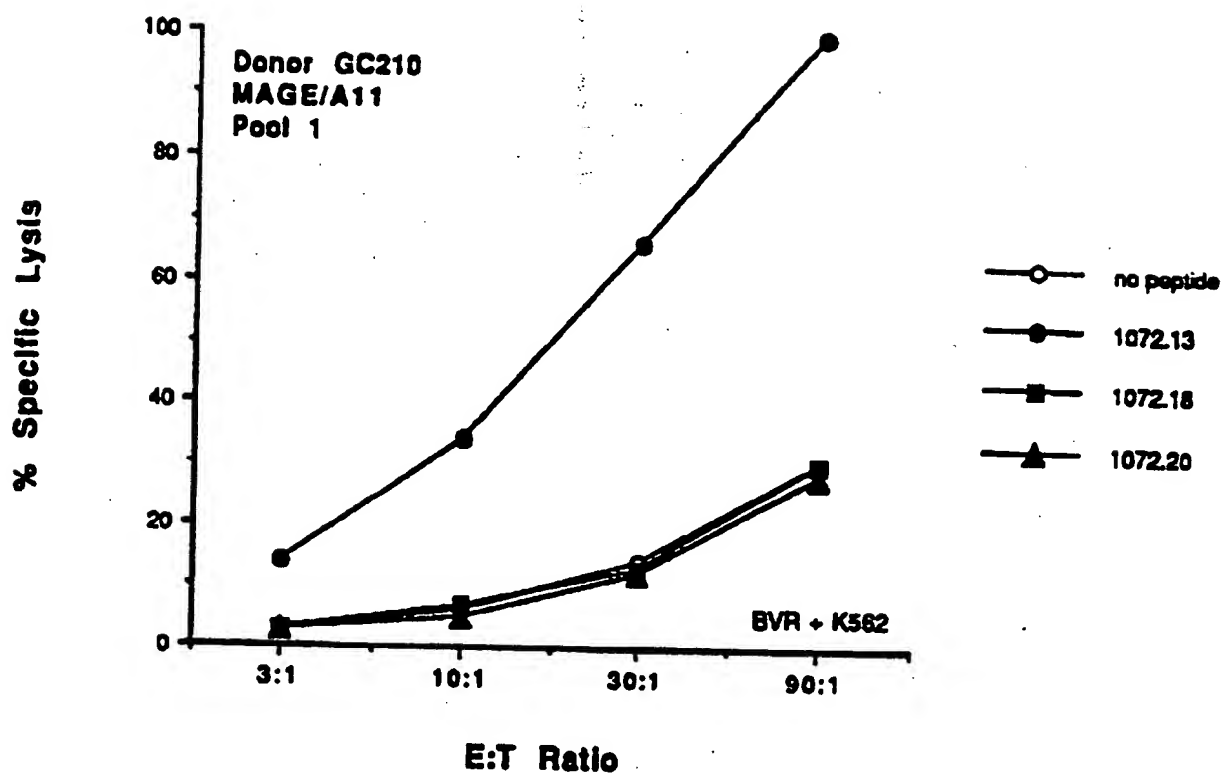
- no peptide
- 1072.39
- 1072.22
- 1072.15

Figure 23

Peptide Screening:
HIV/A3
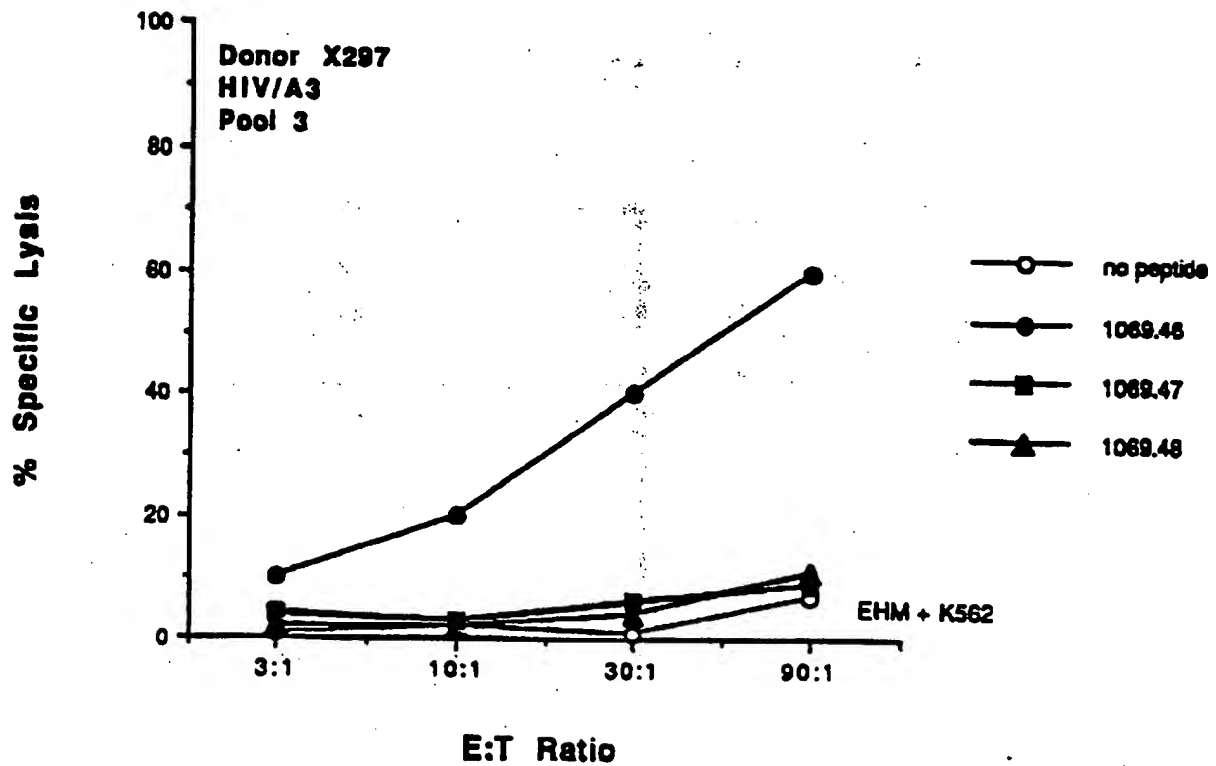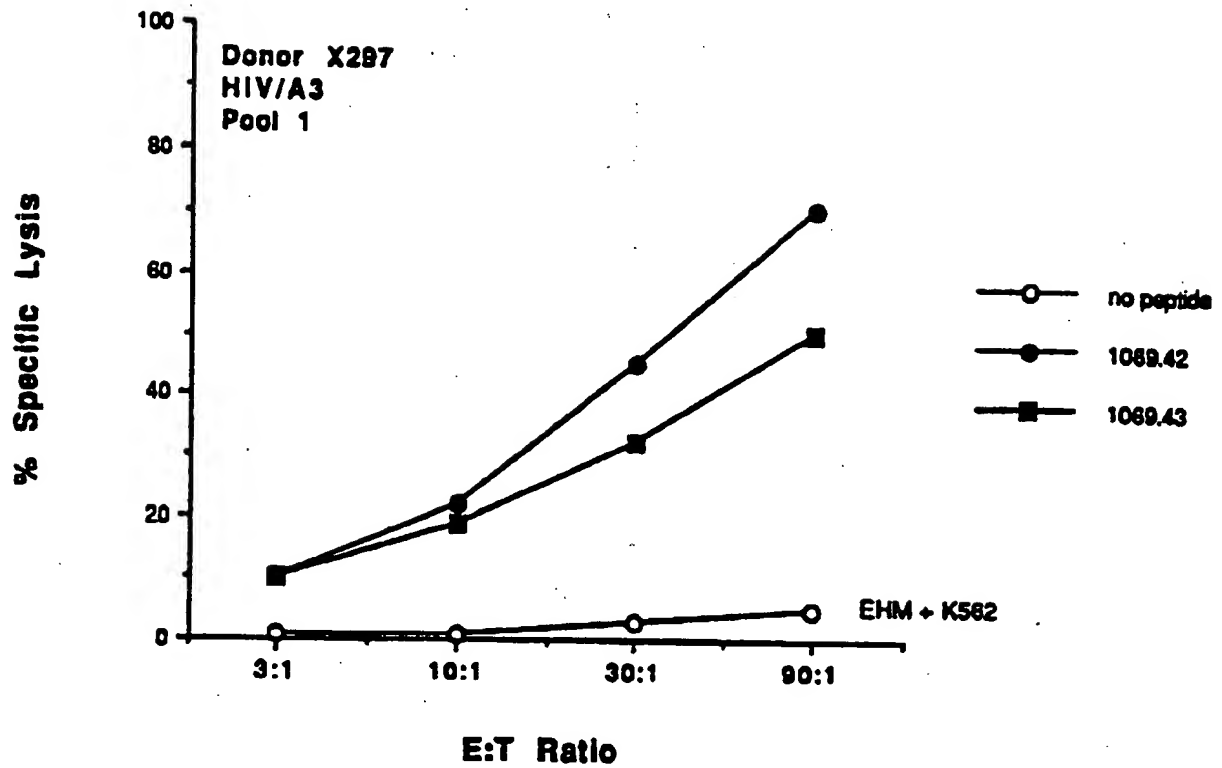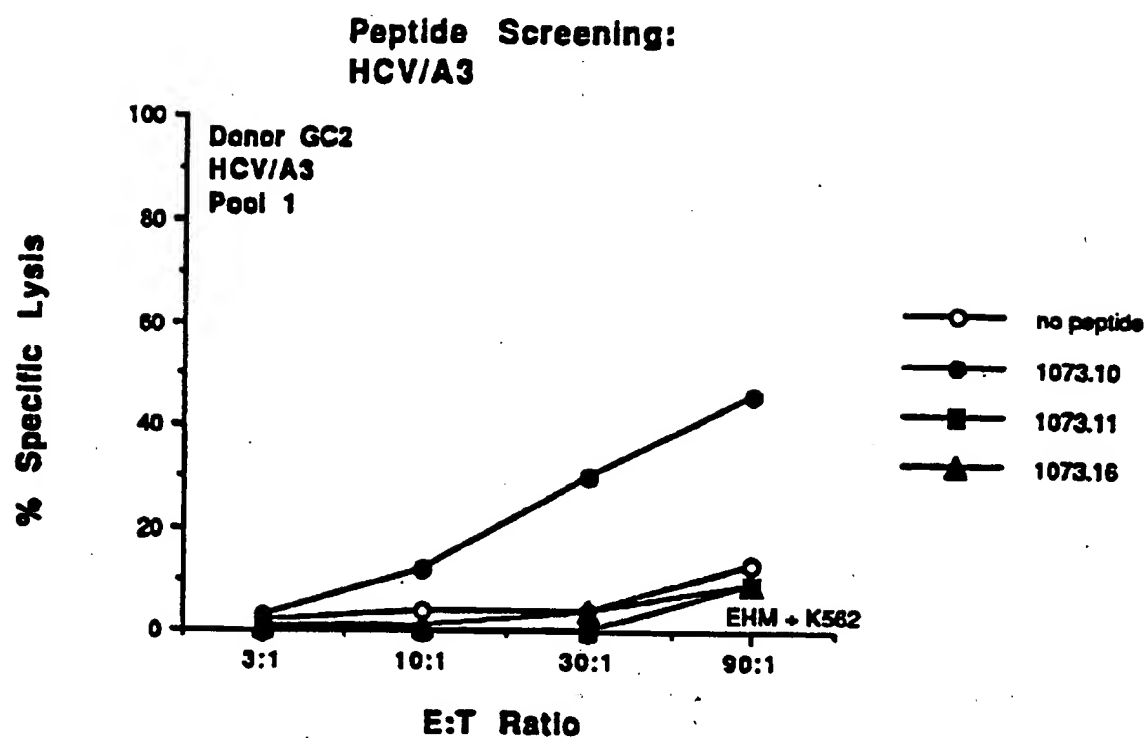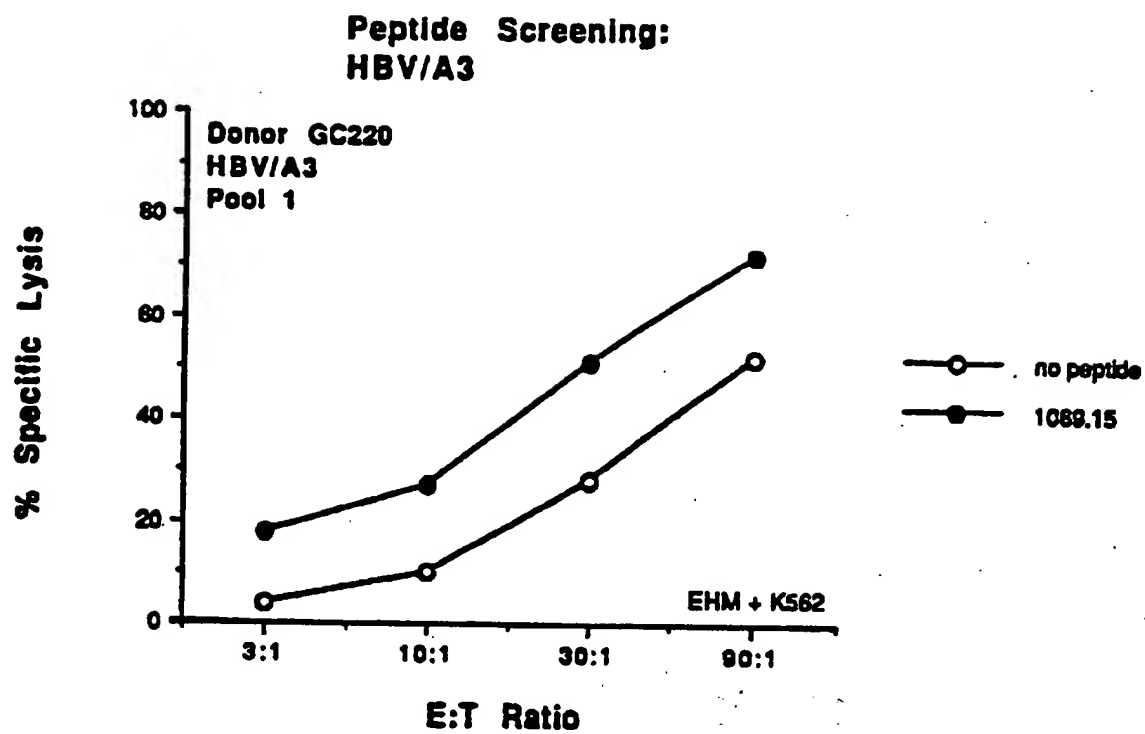
Donor X297
HIV/A3
Pool 1

% Specific Lysis

no peptide
1069.42
1069.43

EHM + K562

E:T Ratio

3:1   10:1   30:1   90:1

Donor X297
HIV/A3
Pool 3

% Specific Lysis

no peptide
1069.46
1069.47
1069.48

EHM + K562

E:T Ratio

3:1   10:1   30:1   90:1

Figure 24

**Peptide   Screening:
HCV/A3**

Figure 25

Peptide Screening:
HBV/A3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00; C07K 7/06, 7/08
US CL : 424/88; 514/2; 530/300, 327, 328

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 514/2; 530/300, 327, 328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., vol. 174, number 3, issued 01 September 1991, Romero, et al., "H2K-d-restricted antigenic peptides share a simple binding motif", pages 603-612, see especially the abstract.	1-20
Y	Nature, vol. 353, issued 26 September 1991, T. S. Jardetzky, et al., "Identification of self peptides bound to purified HLA-B27", pages 326-329, see entire document.	1-20
Y	Nature, vol. 351, issued 23 May 1991, K. Falk, et al., "Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules", pages 290-296, see abstract.	1-20

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 November 1993	17 NOV 1993

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